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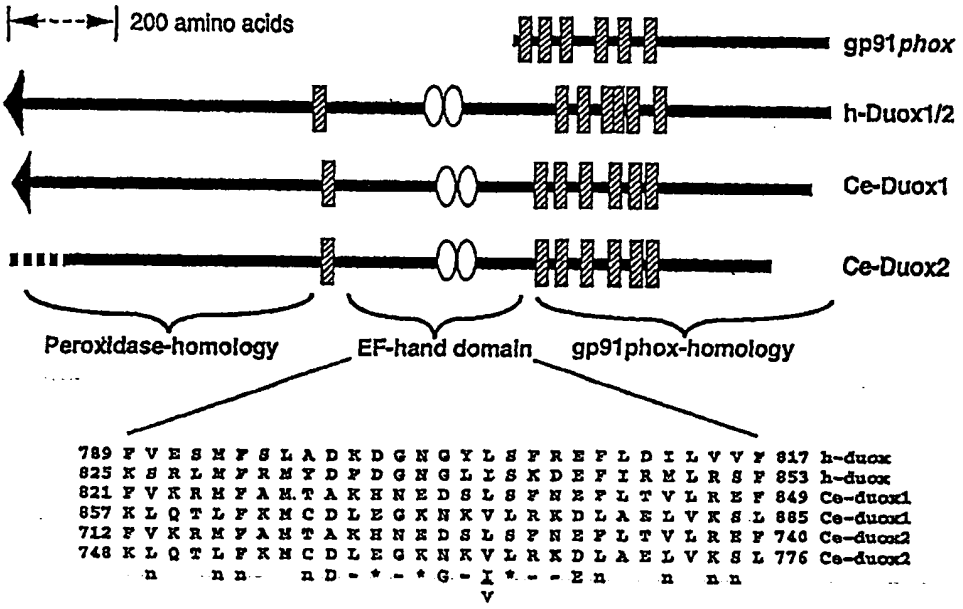
(54) Title: NOVEL DUAL OXIDASES AS MITOGENIC AND ENDOCRINE REGULATORS



(57) Abstract: The present invention relates to new genes encoding for the production of novel proteins involved in generation of reactive oxygen intermediates and in peroxidative reactions that affect biological functions including cell division, thyroid hormone biosynthesis and tissue fibrosis. The present invention also provides vectors containing these genes, cells transfected with these vectors, antibodies raised against these novel proteins, kits for detection, localization and measurement of these genes and proteins, and methods to determine the activity of drugs to affect the activity of the proteins of the present invention.

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NOVEL DUAL OXIDASES AS MITOGENIC AND ENDOCRINE REGULATORS

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TECHNICAL FIELD

The present invention relates to the fields of normal and abnormal cell growth, in particular mitogenic regulation, to thyroid hormone biosynthesis and to nematode cuticle biogenesis. The present invention provides the following: nucleotide sequences encoding for the production of enzymes that are mitogenic regulators, that catalyze thyroid hormone biosynthesis and in nematodes catalyze the biogenesis of cuticle; amino acid sequences of these enzymes; vectors containing these nucleotide sequences; methods for transfecting cells with vectors that produce these enzymes; and antibodies to these enzymes that are useful for detecting and measuring levels of these enzymes, and for binding to cells possessing extracellular epitopes of these enzymes.

BACKGROUND OF THE INVENTION

Reactive oxygen intermediates (ROI) are partial reduction products of oxygen: 1 electron reduces O_2 to form superoxide (O_2^-), and 2 electrons reduce O_2 to form hydrogen peroxide (H_2O_2). ROI are generated as a byproduct of aerobic metabolism and by toxicological mechanisms. There is growing evidence for regulated enzymatic generation of O_2^- and its conversion

to H_2O_2 in a variety of cells. The conversion of O_2^- to H_2O_2 occurs spontaneously, but is markedly accelerated by superoxide dismutase (SOD). High levels of ROI are associated with damage to biomolecules such as DNA, biomembranes and proteins. Recent evidence indicates generation of ROI under normal cellular conditions and points to signaling and metabolic roles for O_2^- and H_2O_2.

Several biological systems generate reactive oxygen. Phagocytic cells such as neutrophils generate large quantities of ROI as part of their battery of bactericidal mechanisms. Exposure of neutrophils to bacteria or to various soluble mediators such as formyl-Met-Leu-Phe or phorbol esters activates a massive consumption of oxygen, termed the respiratory burst, to initially generate superoxide, with secondary generation of H_2O_2, HOCl and hydroxyl radical. The enzyme responsible for this oxygen consumption is the respiratory burst oxidase (nicotinamide adenine dinucleotide phosphate-reduced form (NADPH) oxidase).

There is growing evidence for the generation of ROI by non-phagocytic cells, particularly in situations related to cell proliferation. In addition, in some tissues such as thyroid, ROI generation is implicated in metabolic conversions such as the biosynthesis of thyroid hormone. Significant generation of H_2O_2, O_2^-, or both have been noted in some cell types. Fibroblasts and human endothelial cells show increased release of superoxide in response to cytokines such as interleukin-1 or tumor necrosis factor (TNF) (Meier et al. (1989) *Biochem J.* 263, 539-545.; Matsubara et al. (1986) *J. Immun.* 137, 3295-3298). Ras-transformed fibroblasts show increased superoxide release compared with control fibroblasts (Irani, et al. (1997) *Science* 275, 1649-1652). Rat vascular smooth muscle cells show increased H_2O_2 release in response to PDGF (Sundaresan et al. (1995) *Science* 270, 296-299) and angiotensin II (Griendling et al. (1994) *Circ. Res.* 74, 1141-1148; Fukui et al. (1997) *Circ. Res.* 80, 45-51; Ushio-Fukai et al. (1996) *J. Biol. Chem.* 271, 23317-23321), and H_2O_2 in these cells is associated with increased proliferation rate. The occurrence of ROI in a variety of cell types is summarized in Table 1 (adapted from Burdon, R. (1995) *Free Radical Biol. Med.* 18, 775-794).

Table 1

Superoxide	Hydrogen Peroxide
human fibroblasts	Balb/3T3 cells
human endothelial cells	rat pancreatic isletcells
human/rat smooth muscle cells	murine keratinocytes
human fat cells	rabbit chondrocytes
human osteocytes	human tumor cells
BHK-21 cells	fat cells, 3T3 L1 cells
human colonic epithelial cells	

ROI generated by the neutrophil have a cytotoxic function. While ROI are normally directed at the invading microbe, ROI can also induce tissue damage (e.g., in inflammatory conditions such as arthritis, shock, lung disease, and inflammatory bowel disease) or may be involved in tumor initiation or promotion, due to damaging effects on DNA. Nathan (Szatrowski et al. (1991) *Canc. Res.* 51, 794-798) proposed that the generation of ROI in tumor cells may contribute to the hypermutability seen in tumors, and may therefore contribute to tumor heterogeneity, invasion and metastasis.

In addition to cytotoxic and mutagenic roles, ROI have ideal properties as signal molecules: 1) they are generated in a controlled manner in response to upstream signals; 2) the signal can be terminated by rapid metabolism of O_2^- and H_2O_2 by SOD and catalase/peroxidases; 3) they elicit downstream effects on target molecules, e.g., redox-sensitive regulatory proteins such as NF kappa B and AP-1 (Schreck et al. (1991) *EMBO J.* 10, 2247-2258; Schmidt et al. (1995) *Chemistry & Biology* 2, 13-22). Oxidants such as O_2^- and H_2O_2 have a relatively well defined signaling role in bacteria, operating via the SoxI/II regulon to regulate transcription.

ROI appear to have a direct role in regulating cell division, and may function as mitogenic signals in pathological conditions related to growth. These conditions include cancer and cardiovascular disease. O_2^- is generated in endothelial cells in response to cytokines, and might play a role in angiogenesis (Matsubara et al. (1986) *J. Immun.* 137, 3295-3298). O_2^- and H_2O_2 are also proposed to function as "life-signals", preventing cells from undergoing apoptosis (Matsubara et al. (1986) *J. Immun.* 137, 3295-3298). As discussed above, many cells respond to growth factors (e.g., platelet derived growth factor (PDGF), epidermal derived growth factor (EGF), angiotensin II, and various cytokines) with both increased production of O_2^- /H_2O_2 and increased proliferation. Inhibition of ROI generation prevents the mitogenic response. Exposure to exogenously generated O_2^- and H_2O_2 results in an increase in cell proliferation. A partial list of responsive cell types is shown below in Table 2 (adapted from Burdon, R. (1995) *Free Radical Biol. Med.* 18, 775-794).

Table 2

Superoxide	Hydrogen peroxide
human, hamster fibroblasts	mouse osteoblastic cells
Balb/3T3 cells	Balb/3T3 cells
human histiocytic leukemia	rat, hamster fibroblasts
mouse epidermal cells	human smooth muscle cells
rat colonic epithelial cells	rat vascular smooth muscle
rat vascular smooth muscle cells	cells

While non-transformed cells can respond to growth factors and cytokines with the production of ROI, tumor cells appear to produce ROI in an uncontrolled manner. A series of human tumor cells produced large amounts of hydrogen peroxide compared with non-tumor cells (Szatrowski et al. (1991) *Canc. Res.* 51, 794-798). Ras-transformed NIH 3T3 cells generated elevated amounts of

superoxide, and inhibition of superoxide generation by several mechanisms resulted in a reversion to a "normal" growth phenotype.

O_2^- has been implicated in maintenance of the transformed phenotype in cancer cells including melanoma, breast carcinoma, fibrosarcoma, and virally transformed tumor cells. Decreased levels of the manganese form of SOD (MnSOD) have been measured in cancer cells and *in vitro*-transformed cell lines, predicting increased O_2^- levels (Burdon, R. (1995) *Free Radical Biol. Med.* 18, 775-794). MnSOD is encoded on chromosome 6q25 which is very often lost in melanoma. Overexpression of MnSOD in melanoma and other cancer cells (Church et al. (1993) *Proc. of Natl. Acad. Sci.* 90, 3113-3117; Fernandez-Pol et al. (1982) *Canc. Res.* 42, 609-617; Yan et al. (1996) *Canc. Res.* 56, 2864-2871) resulted in suppression of the transformed phenotype.

ROI are implicated in growth of vascular smooth muscle associated with hypertension, atherosclerosis, and restenosis after angioplasty. O_2^- generation is seen in rabbit aortic adventitia (Pagano et al. (1997) *Proc. Natl. Acad. Sci.* 94, 14483-14488). Vascular endothelial cells release O_2^- in response to cytokines (Matsubara et al. (1986) *J. Immun.* 137, 3295-3298). O_2^- is generated by aortic smooth muscle cells in culture, and increased O_2^- generation is stimulated by angiotensin II which also induces cell hypertrophy. In a rat model system, infusion of angiotensin II leads to hypertension as well as increased O_2^- generation in subsequently isolated aortic tissue (Ushio-Fukai et al. (1996) *J. Biol. Chem.* 271, 23317-23321.; Yu et al. (1997) *J. Biol. Chem.* 272, 27288-27294). Intravenous infusion of a form of SOD that localizes to the vasculature or an infusion of an O_2^- scavenger prevented angiotensin II induced hypertension and inhibited ROI generation (Fukui et al. (1997) *Circ. Res.* 80, 45-51).

The neutrophil NADPH oxidase, also known as phagocyte respiratory burst oxidase, provides a paradigm for the study of the specialized enzymatic ROI-generating system. This extensively studied enzyme oxidizes NADPH and reduces oxygen to form O_2^-. NADPH oxidase consists of multiple proteins and is regulated by assembly of cytosolic and membrane components. The catalytic

moiety consists of flavocytochrome b_{558}, an integral plasma membrane enzyme comprised of two components: gp91phox (gp refers to glycoprotein; phox is an abbreviation of the words phagocyte and oxidase) and p22phox (p refers to protein). gp91phox contains 1 flavin adenine dinucleotide (FAD) and 2 hemes as well as the NADPH binding site.

p22phox has a C-terminal proline-rich sequence which serves as a binding site for cytosolic regulatory proteins. The two cytochrome subunits, gp91phox and p22phox appear to stabilize one another, since the genetic absence of either subunit, as in the inherited disorder chronic granulomatous disease (CGD), results in the absence of the partner subunit (Yu et al. (1997) *J. Biol. Chem.* 272, 27288-27294). Essential cytosolic proteins include p47phox, p67phox and the small GTPase Rac, of which there are two isoforms. p47phox and p67phox both contain SH_3 regions and proline-rich regions which participate in protein interactions governing assembly of the oxidase components during activation. The neutrophil enzyme is regulated in response to bacterial phagocytosis or chemotactic signals by phosphorylation of p47phox, and perhaps other components, as well as by guanine nucleotide exchange to activate the GTP-binding protein Rac.

The origin of ROI in non-phagocytic tissues is unproven, but the occurrence of phagocyte oxidase components has been evaluated in several systems by immunochemical methods, Northern blots and reverse transcriptase-polymerase chain reaction (RT-PCR). The message for p22phox is expressed widely, as is that for Rac1. Several cell types that are capable of O_2^- generation have been demonstrated to contain all of the phox components including gp91phox, as summarized below in Table 3. These cell types include endothelial cells, aortic adventitia and lymphocytes.

Table 3

Tissue	gp91phox	p22phox	p47phox	p67phox
neutrophil	$+^{1,2}$	$+^{1,2}$	$+^{1,2}$	$+^{1,2}$
aortic adventitia	$+^{1}$	$+^{1}$	$+^{1}$	$+^{1}$
lymphocytes	$+^{2}$	$+^{2}$	$+^{1,2}$	$+^{1,2}$
endothelial cells	$+^{2}$	$+^{2}$	$+^{1,2}$	$+^{1,2}$
glomerular mesangial cells	-	$+^{1,2}$	$+^{1,2}$	$+^{1,2}$
fibroblasts	-	$+^{2}$	$+^{1,2}$	$+^{2}$
aortic sm. muscle	-	$+^{1,2}$	?	?

1= protein expression shown. 2= mRNA expression shown.

A distinctly different pattern is seen in several other cell types shown in Table 3 including glomerular mesangial cells, rat aortic smooth muscle and fibroblasts. In these cells, expression of gp91phox is absent while p22phox and in some cases cytosolic phox components have been demonstrated to be present. Since gp91phox and p22phox stabilize one another in the neutrophil, there has been much speculation that some molecule, possibly related to gp91phox, accounts for ROI generation in glomerular mesangial cells, rat aortic smooth muscle and fibroblasts (Ushio-Fukai et al. (1996) *J. Biol. Chem.* 271, 23317-23321). Investigation of fibroblasts from a patient with a genetic absence of gp91phox provides proof that the gp91phox subunit is not involved in ROI generation in these cells (Emmendorffer et al. (1993) *Eur. J. Haematol.* 51, 223-227). Depletion of p22phox from vascular smooth muscle using an antisense approach indicated that this subunit participates in ROI generation in these cells, despite the absence of detectable gp91phox (Ushio-Fukai et al. (1996) *J. Biol. Chem.* 271, 23317-23321).

Thyroid hormone regulates basal metabolic rate through end-effects on mitochondrial respiration, and conditions of under- or over-production are important clinically. Development of drugs to regulate the biosynthesis of thyroid hormone is a medically important goal, and identification of the enzymes in this pathway is key to developing

pharmacologically relevant targets. Thyroid uniquely concentrates iodide, which is used to iodinate tyrosine residues on thyroglobulin (TG). TG is a large protein (660 kDa) that contains 67 tyrosyl residues, some of which are preferential sites for iodination. Iodination of tyrosines in TG is catalyzed by thyroid peroxidase (TPO), a plasma membrane hemoprotein. Iodination requires a previously unidentified enzymatic source of H_2O_2. A second step is the coupling of two diiodotyrosines (DIT) to form protein-associated thyroxine (T4), which is subsequently proteolytically cleaved from TG to liberate free T4. What is needed is a composition of the gene that encodes the enzyme that generates H_2O_2 in thyroid and that catalyzes the coupling reaction, and a method of using that composition to modulate thyroid hormone biosynthesis. Such information would be useful in the development of drugs for modulation of thyroid function. Such modulation might be useful in the treatment of hyperthyroidism.

Recent evidence suggests that enzymes involved in oxidative cross-linking of tyrosine in growth factor stimulated fibroblasts may lead to fibrotic damage. Lung fibrosis is particularly damaging to individuals afflicted with this condition. Identification of the genes encoding enzymes involved in such oxidative cross-linking reactions is needed so that drugs may be designed to alleviate or prevent fibrotic damage, particularly in the lung.

Parasitic diseases are a major cause of morbidity and mortality worldwide in humans and animals, and have a significant impact on agricultural productivity as well. Parasitic diseases have proven difficult to treat, in part due to the presence of the cuticle, a tough exoskeletal structure of parasites such as nematodes. What is needed is a composition and method of using the composition to fight parasitic diseases, including but not limited to those parasitic diseases caused by parasites with cuticles.

Accordingly, what is needed is a method of disrupting the formation of the cuticle which would make the worm susceptible to the host defense mechanisms and drug treatment.

What is also needed is the identity of the proteins involved in ROI generation, especially in non-phagocytic tissues and

cells. What is also needed are the nucleotide sequences encoding for these proteins, and the primary sequences of the proteins themselves. Also needed are vectors designed to include nucleotides encoding for these proteins. Probes and PCR primers derived from the nucleotide sequence are needed to detect, localize and measure nucleotide sequences, including mRNA, involved in the synthesis of these proteins. In addition, what is needed is a means to transfect cells with these vectors. What is also needed are expression systems for production of these molecules. Also needed are antibodies directed against these molecules for a variety of uses including localization, detection, and measurement and passive immunization.

SUMMARY OF THE INVENTION

The present invention solves the problems described above by providing a novel family of nucleotide sequences and proteins encoded by these nucleotide sequences termed duox proteins. In particular, the present invention provides compositions comprising the nucleotide sequences SEQ ID NO: 1 and SEQ ID NO: 3, and fragments and conservative substitutions thereof, which encode for the expression of proteins comprising SEQ ID NO: 2 and SEQ ID NO: 4 respectively, and fragments and conservative substitutions thereof. Preferred protein fragments include, but are not limited to, SEQ ID NO: 31 and SEQ ID NO: 32. While not wanting to be bound by the following statement, it is believed that these proteins are involved in ROI production and are capable of stimulating superoxide production or generating peroxidative reactions. The present invention also provides vectors containing these nucleotide sequences, cells transfected with these vectors which produce the proteins comprising SEQ ID NO: 2 and SEQ ID NO: 4, and fragments and conservative substitutions thereof, and antibodies to these proteins and fragments and conservative substitutions thereof. The present invention also provides methods for stimulating cellular proliferation by administering vectors encoded for production of the proteins comprising SEQ ID NO: 2 and SEQ ID NO: 4 and fragments and conservative substitutions thereof. The present invention also provides methods for stimulating cellular proliferation by

administering the proteins comprising SEQ ID NO: 2 and SEQ ID NO: 4 and fragments and conservative substitutions thereof. The proteins comprising SEQ ID NO: 2 and SEQ ID NO: 4 and fragments and conservative substitutions thereof are useful in affecting the exoskeleton, especially the cuticle of parasites, including but not limited to nematodes. The nucleotides and antibodies of the present invention are useful for the detection, localization and measurement of the nucleic acids encoding for the production of the proteins of the present invention, and also for the detection, localization and measurement of the proteins of the present invention. These nucleotides and antibodies can be combined with other reagents in kits for the purposes of detection, localization and measurement.

Most particularly, the present invention involves a method for regulation of cell division or cell proliferation by modifying the activity or expression of the duox proteins described as SEQ ID NO: 2 and SEQ ID NO: 4 or fragments or conservative substitutions thereof. These proteins, in their naturally occurring or expressed forms, are expected to be useful in drug development, for example for screening of chemical and drug libraries by observing inhibition of the activity of these enzymes. Such chemicals and drugs would likely be useful as treatments for cancer, prostatic hypertrophy, benign prostatic hypertrophy, hypertension, metabolic disease, fibrosis, atherosclerosis and many other disorders involving abnormal cell growth or proliferation, and a variety of parasitic diseases in both animals and crops as described below. The entire expressed protein may be useful in these assays. Portions of the molecule which may be targets for inhibition or modification include but are not limited to the binding site for pyridine nucleotides (NADPH or NADH), the flavoprotein domain (approximately the C-terminal 265 amino acids), and/or the binding or catalytic site for flavin adenine dinucleotide (FAD).

The method of the present invention may be used for the development of drugs or other therapies for the treatment of conditions associated with abnormal growth including, but not limited to the following: cancer, fibrosis, lung fibrosis, metabolic imbalances, thyroid imbalances, hyperthryoidism, psoriasis, prostatic hypertrophy,

benign prostatic hypertrophy, cardiovascular disease, proliferation of vessels, including but not limited to blood vessels and lymphatic vessels, arteriovenous malformation, vascular problems associated with the eye, atherosclerosis, hypertension, and restenosis following angioplasty and parasitic diseases. The enzymes of the present invention are excellent targets for the development of drugs and other agents which may modulate the activity of these enzymes. It is to be understood that modulation of activity may result in enhanced, diminished or absence of enzymatic activity. Modulation of the activity of these enzymes may be useful in treatment of conditions, including but not limited to conditions associated with abnormal growth, metabolic disorders, and fibrosis.

Drugs which affect the activity of the duox enzymes represented in SEQ ID NO: 2 and SEQ ID NO: 4, or fragments or conservative substitutions thereof, may also be combined with other therapeutics in the treatment of specific conditions. For example, these drugs may be combined with angiogenesis inhibitors in the treatment of cancer, with antihypertensives for the treatment of hypertension, with cholesterol lowering drugs for the treatment of atherosclerosis and with hormonal agonists or antagonists in the treatment of endocrine disorders, such as thyroid disorders.

It is to be understood that the proteins of the present invention, including but not limited to, SEQ ID NO: 2 and SEQ ID NO: 4, or fragments or conservative substitutions thereof, may be administered together with other compositions such as anti-parasitic compositions, pesticides, herbicides and fertilizers. Accordingly, the proteins of the present invention may be useful alone or in combination with other compositions for treating humans or animals, including livestock, other farm animals and domestic animals, including pets, for preventing or fighting parasitic disease, for protecting plants and crops against attack by parasites, especially soil nematodes, and for destroying parasites.

Accordingly, an object of the present invention is to provide nucleotide sequences, or fragments thereof or conservative substitutions thereof, encoding for the production of proteins, or fragments thereof or conservative substitutions thereof, that are

involved in ROI production, stimulate superoxide production or generate peroxidative reactions.

It is another object of the present invention is to provide the proteins represented in SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 31, and SEQ ID NO: 32 or fragments or conservative substitutions thereof.

It is another object of the present invention is to provide the nucleotide sequences encoding for the proteins represented in SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 31, and SEQ ID NO: 32 or fragments or conservative substitutions thereof, wherein these nucleotide sequence include SEQ ID NO: 1, SEQ ID NO: 3 or fragments or conservative substitutions thereof.

It is another object of the present invention to provide proteins, fragments thereof or conservative substitutions thereof, involved in exoskeletal or cuticle formation that may be used as targets for therapies designed to prevent exoskeletal or cuticle formation and to harm organisms having an exoskeleton or cuticle, particularly parasites.

It is another object of the present invention to provide proteins, fragments thereof or conservative substitutions thereof, involved in thyroid hormone biosynthesis that may be used as targets for therapies designed to inhibit biosynthesis of thyroid hormone.

It is yet another object of the present invention to provide proteins, fragments thereof or conservative substitutions thereof, involved in tissue fibrosis that may be used as targets for therapies designed to prevent fibrosis.

Another object of the present invention is to provide proteins involved in lung fibrosis that may be used as targets for therapies designed to prevent lung fibrosis.

Another object of the present invention is to provide vectors containing these nucleotide sequences, or fragments thereof.

Yet another object of the present invention is to provide cells transfected with these vectors.

Still another object of the present invention is to administer cells transfected with these vectors to animals and humans.

Another object of the present invention is to provide proteins, or fragments thereof, that are involved in ROI production, stimulate superoxide production or generate peroxidative reactions.

Still another object of the present invention is to provide antibodies, including monoclonal and polyclonal antibodies, or fragments thereof, raised against proteins, or fragments thereof or conservative substitutions thereof, that are involved in ROI production, stimulate superoxide production or generate peroxidative reactions. Such antibodies are useful in the localization and measurement of proteins, or fragments thereof, that are involved in ROI production.

Another object of the present invention is to administer genes containing nucleotide sequences, or fragments thereof, encoding for the production of proteins, or fragments thereof or conservative substitutions thereof, that are involved in ROI production, stimulate superoxide production or generate peroxidative reactions, to animals and humans and also to cells obtained from animals and humans.

Another object of the present invention is to administer antisense complimentary sequences of genes containing nucleotide sequences, or fragments thereof or conservative substitutions thereof, encoding for the production of proteins, or fragments thereof or conservative substitutions thereof, that are involved in ROI production, stimulate superoxide production or generate peroxidative reactions, to animals and humans and also to cells obtained from animals and humans.

Yet another object of the present invention is to provide a method for stimulating or inhibiting cellular proliferation by administering vectors containing nucleotide sequences, or fragments thereof, encoding for the production of proteins, or fragments thereof, that are involved in ROI production, stimulate superoxide production or generate peroxidative reactions, to animals and humans. It is also an object of the present invention to provide a method for stimulating or inhibiting cellular proliferation by administering vectors containing antisense complimentary sequences of nucleotide sequences, or fragments thereof, encoding for the production of proteins, or fragments thereof, that are involved in ROI production, stimulate

superoxide production or generate peroxidative reactions, to animals and humans. These methods of stimulating cellular proliferation are useful for a variety of purposes, including but not limited to, developing animal models of tumor formation, stimulating cellular proliferation of blood marrow cells following chemotherapy or radiation, or in cases of anemia.

Yet another object of the present invention is to provide nucleotide probes useful for the detection, localization and measurement of nucleotide sequences, or fragments thereof, encoding for the production of proteins, or fragments thereof, that are involved in ROI production, stimulate superoxide production or generate peroxidative reactions.

Another object of the present invention is to provide kits useful for detection of nucleic acids including the nucleic acids represented in SEQ ID NO: 1, and SEQ ID NO: 3, or fragments thereof or conservative substitutions thereof, that encode for proteins, or fragments thereof or conservative substitutions thereof, that are involved in ROI production, stimulate superoxide production or generate peroxidative reactions.

Yet another object of the present invention is to provide kits useful for detection and measurement of nucleic acids including the nucleic acids represented in SEQ ID NO: 1, and SEQ ID NO: 3, or fragments thereof, that encode for proteins, or fragments thereof or conservative substitutions thereof, that are involved in ROI production, stimulate superoxide production or generate peroxidative reactions.

Another object of the present invention is to provide kits useful for detection of proteins, including the proteins represented in SEQ ID NO: 2 and SEQ ID NO: 4 or fragments thereof, that are involved in ROI production, stimulate superoxide production or generate peroxidative reactions.

Yet another object of the present invention is to provide kits useful for detection and measurement of proteins, including the proteins represented in SEQ ID NO: 2 and SEQ ID NO: 4, or fragments thereof, that are involved in ROI production, stimulate superoxide production or generate peroxidative reactions.

Still another object of the present invention is to provide kits useful for localization of proteins, including the proteins represented in SEQ ID NO: 2 and SEQ ID NO: 4, or fragments thereof, that are involved in ROI production, stimulate superoxide production or generate peroxidative reactions.

Yet another object of the present invention is to provides kits useful for the detection, measurement or localization of nucleic acids, or fragments thereof, encoding for proteins, or fragments thereof, that are involved in ROI production, for use in diagnosis and prognosis of abnormal cellular proliferation related to ROI production.

Another object of the present invention is to provides kits useful for the detection, measurement or localization of proteins, or fragments thereof, that are involved in ROI production, for use in diagnosis and prognosis of abnormal cellular proliferation related to ROI production.

These and other objects, features and advantages of the present invention will become apparent after a review of the following detailed description of the disclosed embodiments and the appended drawings.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Structure of large homologs of gp91*phox*. Domain structure of Duox proteins. Secretory signal peptide sequences are indicated by a gray triangle, while predicted transmembrane alpha helices are indicated by a hashed rectangle. White ovals indicate regions showing homology with EF-hand calcium binding sites.

Figure 2. Comparison of the peroxidase domains of h-Duox, Ce-Duox1 and some known peroxidases.

A) Sequence alignments. Abbreviations are: MPO, myeloperoxidase; TPO, thyroid peroxidase; EPO, eosinophil peroxidase; LPO, lactoperoxidase, Pxsn.dros, *Drosophila* peroxidasin. Residues which are conserved among all 7 proteins are shown with black boxes, while those matching a derived consensus sequence are shown in line boxes. Filled circles indicate residues which are

proposed to provide contacts with the heme, based on the crystal structure of canine myeloperoxidase (Zeng and Fenna, 1992). The superscripted double bar indicates residues comprising a calcium binding region, and filled triangles indicate residues which appear in the crystal structure to bind directly to the calcium ion.

B) Phylogenetic relationships. The sequences shown in A as well as additional sequences are shown. Abbreviations are: OPO, ovoperoxidase; str.purp, *Strongylocentrotus purpuratus*; ly.var, *Lytechinus variegatus*; hemi.pulch; *Hemicentrotus pulcherrimus*.

Figure 3. Tissue expression of mRNA for h-Duox. mRNA for h-Duox1, h-Duox2 and glyceraldehyde 3-phosphate dehydrogenase was detected by RT-PCR.

DETAILED DESCRIPTION OF THE INVENTION

The present invention solves the problems described above by providing a novel family of nucleotide sequences and proteins, encoded by these nucleotide sequences, termed duox proteins. The term "duox" refers to "dual oxidase". In particular, the present invention provides novel compositions comprising the nucleotide sequences SEQ ID NO: 1, and SEQ ID NO: 3, and fragments thereof or conservative substitutions thereof, which encode, respectively, for the expression of proteins comprising SEQ ID NO: 2 and SEQ ID NO: 4 and fragments thereof or conservative substitutions thereof. Preferred protein fragments include, but are not limited to SEQ ID NO: 31 and SEQ ID NO: 32.

The duox proteins described herein have homology to the gp91phox protein involved in ROI generation, however, the duox proteins comprise a novel and distinct family of proteins. The duox proteins described herein have three distinct regions: the amino terminal region having homology to peroxidase proteins, the internal region having homology to calmodulin (CAM) proteins and the carboxy-terminal region having homology to mox (also called nox) proteins. The amino acid sequence of human duox2 is shown in SEQ ID NO: 2. Nucleotides encoding duox2 proteins are also shown in

SEQ ID NO: 1. In addition to the human duox proteins, comparison of the sequence of human duox1 and human duox2 with genomic databases using BLAST searching resulted in the identification of two homologs of duox in C. elegans (Ce-duox1 SEQ ID NO: 3) and the pseudogene Ce-duox2. Drosophila also appears to have at least one duox homolog. Thus, the duox family of genes/proteins is widely distributed among multicellular organisms.

High molecular weight homologs of gp91*phox*, have been identified in human (h) and *C. elegans* (Ce), and are termed Duox for "dual oxidase" because they have both a peroxidase-homology domain and a gp91*phox* domain. Ce-Duox uses cytosolic NADPH to generate reactive oxygen. It catalyzes cross-linking of free tyrosine ethyl ester involved in the stabilization of the cuticular extracellular matrix in nematodes.

Although not wanting to be bound by the following statement, it is believed that duox enzymes, for example duox2 and Ce-duox1, have dual enzymatic functions, catalyzing both the generation of superoxide and peroxidative type reactions. The latter class of reactions utilize hydrogen peroxide as a substrate (and in some cases have been proposed to utilize superoxide as a substrate). Since hydrogen peroxide is generated spontaneously from the dismutation of superoxide, it is believed that the NAD(P)H oxidase domain generates the superoxide and/or hydrogen peroxide which can then be used as a substrate for the peroxidase domain. In support of this hypothesis, a model for the duox2 protein in *C. elegans* has been developed that has an extracellular N-terminal peroxidase domain, a transmembrane region and a NADPH binding site located on the cytosolic face of the plasma membrane. By analogy with the neutrophil NADPH-oxidase which generates extracellular superoxide, human duox2 is predicted to generate superoxide and its byproduct hydrogen peroxide extracellularly where it can be utilized by the peroxidase domain.

The peroxidase domain is likely to confer additional biological functions. Depending upon the co-substrate, peroxidases can participate in a variety of reactions including halogenation such as the generation of hypochlorous acid (HOCl) by myeloperoxidase and

the iodination of tyrosine to form thyroxin by thyroid peroxidase. Peroxidases have also been documented to participate in the metabolism of polyunsaturated fatty acids, and in the chemical modification of tyrosine in collagen (by sea urchin ovoperoxidase). Although not wanting to be bound by this statement, it is believed that the predicted transmembrane nature of duox2 facilitates its function in the formation or modification of extracellular matrix or basement membrane. Since the extracellular matrix plays an important role in tumor cell growth, invasion and metastasis, it is believed that the duox type enzymes play a pathogenic role in such conditions.

In addition to the nucleotide sequences described above, the present invention also provides vectors containing these nucleotide sequences and fragments thereof or conservative substitutions thereof, cells transfected with these vectors which produce the proteins comprising SEQ ID NO: 2 and SEQ ID NO: 4 and fragments thereof or conservative substitutions thereof, and antibodies to these proteins and fragments thereof. The present invention also provides methods for stimulating cellular proliferation by administering vectors, or cells containing vectors, encoded for production of the proteins comprising SEQ ID NO: 2, SEQ ID NO: 4, and fragments thereof. The nucleotides and antibodies of the present invention are useful for the detection, localization and measurement of the nucleic acids encoding for the production of the proteins of the present invention, and also for the detection, localization and measurement of the proteins of the present invention. These nucleotides and antibodies can be combined with other reagents in kits for the purposes of detection, localization and measurement. These kits are useful for diagnosis and prognosis of conditions involving cellular proliferation associated with production of reactive oxygen intermediates.

The present invention solves the problems described above by providing a composition comprising the nucleotide sequence SEQ ID NO: 1 and fragments thereof and conservative substitutions thereof. The present invention also provides a composition comprising the nucleotide sequence SEQ ID NO: 3 and fragments thereof and conservative substitutions thereof. The present invention

provides a composition comprising the protein SEQ ID NO: 2, and fragments and conservative substitutions thereof, encoded by the nucleotide sequence SEQ ID NO: 1 and fragments and conservative substitutions thereof. The present invention provides a composition comprising the protein SEQ ID NO: 4 and fragments and conservative substitutions thereof, encoded by the nucleotide sequence SEQ ID NO: 3 and fragments and conservative substitutions thereof. Preferred protein fragments include, but are not limited to, SEQ ID NO: 31 and SEQ ID NO: 32.

The present invention also provides vectors containing the nucleotide sequences SEQ ID NO: 1, and SEQ ID NO: 3 or fragments thereof. The present invention also provides cells transfected with these vectors.

In addition, the present invention provides cells stably transfected with the nucleotide sequence SEQ ID NO: 1 or fragments thereof. The present invention also provides cells stably transfected with the nucleotide sequence SEQ ID NO: 3 or fragments thereof.

The present invention provides cells stably transfected with the nucleotide sequence SEQ ID NO: 1 or fragments or conservative substitutions thereof, which produce the protein SEQ ID NO: 2 or fragments or conservative substitutions thereof. In addition, the present invention provides cells stably transfected with the nucleotide sequence SEQ ID NO: 3 or fragments or conservative substitutions thereof which produce the protein SEQ ID NO: 4 or fragments or conservative substitutions thereof.

The present invention provides a method for stimulating growth by administering cells stably transfected with the nucleotide sequence SEQ ID NO: 1 or fragments or conservative substitutions thereof which produce the protein SEQ ID NO: 2 or fragments or conservative substitutions thereof. The present invention also provides a method for stimulating growth by administering cells stably transfected with the nucleotide sequence SEQ ID NO: 3 or fragments or conservative substitutions thereof, which produce the protein SEQ ID NO: 4 or fragments or conservative substitutions thereof.

Specifically, the present invention provides a method for stimulating tumor formation by administering cells stably transfected with the nucleotide sequence SEQ ID NO: 1 or fragments thereof, which produce the protein SEQ ID NO: 2 or fragments thereof. The present invention also provides a method for stimulating tumor formation by administering cells stably transfected with the nucleotide sequence SEQ ID NO: 3 or fragments thereof, which produce the protein SEQ ID NO: 4 or fragments thereof. The present invention may also be used to develop anti-sense nucleotide sequences to SEQ ID NO: 1 and SEQ ID NO: 3, or fragments thereof. These anti-sense molecules may be used to interfere with translation of nucleotide sequences, such as SEQ ID NO: 1, and SEQ ID NO: 3, or fragments thereof, that encode for proteins such as SEQ ID NO: 2, SEQ ID NO: 4, or fragments thereof. Administration of these anti-sense molecules, or vectors encoding for anti sense molecules, to humans and animals, would interfere with production of proteins such as SEQ ID NO: 2, SEQ ID NO: 4, or fragments thereof, thereby decreasing production of ROIs and inhibiting cellular proliferation. These methods are useful in producing animal models for use in study of tumor development, cuticle formation and vascular growth, and for study of the efficacy of treatments for affecting tumor growth, vascular growth and cuticle formation *in vivo*.

The present invention also provides a method for high throughput screening of drugs and chemicals which modulate the proliferative activity of the enzymes of the present invention or fragments or conservative substitutions thereof, thereby affecting cell division, metabolic activity, cuticle formation, fibrosis and other biological functions involving oxidative reactions. Combinatorial chemical libraries may be screened for chemicals which modulate the proliferative activity or oxidative activity of these enzymes. Drugs and chemicals may be evaluated based on their ability to modulate the enzymatic activity of the expressed or endogenous proteins, including those represented SEQ ID NO: 2 and SEQ ID NO: 4 or fragments or conservative substitutions thereof. Endogenous proteins may be obtained from many different tissues or cells, such as colon cells. Drugs may also be evaluated based on their ability to bind to the

expressed or endogenous proteins represented by SEQ ID NO: 2 and SEQ ID NO: 4 or fragments or conservative substitutions thereof. Enzymatic activity may be NADPH- or NADH-dependent superoxide generation catalyzed by the holoprotein. Enzymatic activity may also be NADPH- or NADH-dependent diaphorase activity catalyzed by either the holoprotein or the flavoprotein domain.

By flavoprotein domain, is meant approximately the C-terminal half of the enzymes shown in SEQ ID NO: 2 and SEQ ID NO: 4, or fragments or conservative substitutions thereof, (approximately the C-terminal 265 amino acids). This fragment of gp91phox has NADPH-dependent reductase activity towards cytochrome c, nitrobluetetrazolium and other dyes. Expressed proteins or fragments thereof can be used for robotic screens of existing combinatorial chemical libraries. While not wanting to be bound by the following statement, it is believed that the NADPH or NADH binding site and the FAD binding site are useful for evaluating the ability of drugs and other compositions to bind to the duox enzymes or fragments or conservative substitutions thereof, or to modulate their enzymatic activity. The use of the holoprotein or the C-terminal half or end regions are preferred for developing a high throughput drug screen. Additionally, the N-terminal one-third of the duox domain (the peroxidase domain) may also be used to evaluate the ability of drugs and other compositions to inhibit the peroxidase activity, and for further development of a high throughput drug screen.

The present invention also provides antibodies directed to the oxidative enzymes such as SEQ ID NO: 2 and SEQ ID NO: 4 and fragments or conservative substitutions thereof. Preferred protein fragments include, but are not limited to, SEQ ID NO: 31 and SEQ ID NO: 32. The antibodies of the present invention are useful for a variety of purposes including localization, detection and measurement of the proteins SEQ ID NO: 2 and SEQ ID NO: 4 and fragments or conservative substitutions thereof. The antibodies may be employed in kits to accomplish these purposes. These antibodies may also be linked to cytotoxic agents for selected killing of cells. The term antibody is meant to include any class of antibody such as IgG, IgM

and other classes. The term antibody also includes a completely intact antibody and also fragments thereof, including but not limited to Fab fragments and Fab + Fc fragments.

The present invention also provides the nucleotide sequences SEQ ID NO: 1 and SEQ ID NO: 3 and fragments or conservative substitutions thereof. These nucleotides are useful for a variety of purposes including localization, detection, and measurement of messenger RNA involved in synthesis of the proteins represented as SEQ ID NO: 2 and SEQ ID NO: 4 and fragments or conservative substitutions thereof. These nucleotides may also be used in the construction of labeled probes for the localization, detection, and measurement of nucleic acids such as messenger RNA or alternatively for the isolation of larger nucleotide sequences containing the nucleotide sequences shown in SEQ ID NO: 1, and SEQ ID NO: 3 or fragments or conservative substitutions thereof. These nucleotide sequences may be used to isolate homologous strands from other species using techniques known to one of ordinary skill in the art. These nucleotide sequences may also be used to make probes and complementary strands.

Most particularly, the present invention involves a method for modulation of growth by modifying the proteins represented as SEQ ID NO: 2 and SEQ ID NO: 4 or fragments or conservative substitutions thereof.

The term "mitogenic regulators" is used herein to mean any molecule that acts to affect cell division.

The term "animal" is used herein to mean humans and non-human animals of both sexes.

The terms "a", "an" and "the" as used herein are defined to mean one or more and include the plural unless the context is inappropriate.

"Proteins", "peptides," "polypeptides" and "oligopeptides" are chains of amino acids (typically L-amino acids) whose alpha carbons are linked through peptide bonds formed by a condensation reaction between the carboxyl group of the alpha carbon of one amino acid and the amino group of the alpha carbon of another amino acid. The terminal amino acid at one end of the chain (*i.e.*, the

amino terminal) has a free amino group, while the terminal amino acid at the other end of the chain (*i.e.*, the carboxy terminal) has a free carboxyl group. As such, the term "amino terminus" (abbreviated N-terminus) refers to the free alpha-amino group on the amino acid at the amino terminal of the protein, or to the alpha-amino group (imino group when participating in a peptide bond) of an amino acid at any other location within the protein. Similarly, the term "carboxy terminus" (abbreviated C-terminus) refers to the free carboxyl group on the amino acid at the carboxy terminus of a protein, or to the carboxyl group of an amino acid at any other location within the protein.

Typically, the amino acids making up a protein are numbered in order, starting at the amino terminal and increasing in the direction toward the carboxy terminal of the protein. Thus, when one amino acid is said to "follow" another, that amino acid is positioned closer to the carboxy terminal of the protein than the preceding amino acid.

The term "residue" is used herein to refer to an amino acid (D or L) or an amino acid mimetic that is incorporated into a protein by an amide bond. As such, the amino acid may be a naturally occurring amino acid or, unless otherwise limited, may encompass known analogs of natural amino acids that function in a manner similar to the naturally occurring amino acids (*i.e.*, amino acid mimetics). Moreover, an amide bond mimetic includes peptide backbone modifications well known to those skilled in the art.

Furthermore, one of skill will recognize that, as mentioned above; individual substitutions, deletions or additions which alter, add or delete a single amino acid or a small percentage of amino acids (typically less than 5%, more typically less than 1%) in an encoded sequence are conservatively modified variations where the alterations result in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art. The following six groups each contain amino acids that are conservative substitutions for one another:

1) Alanine (A), Serine (S), Threonine (T);

2) Aspartic acid (D), Glutamic acid (E);

3) Asparagine (N), Glutamine (Q);

4) Arginine (R), Lysine (K);

5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and

6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

When the peptides are relatively short in length (*i.e.*, less than about 50 amino acids), they are often synthesized using standard chemical peptide synthesis techniques. Solid phase synthesis in which the C-terminal amino acid of the sequence is attached to an insoluble support followed by sequential addition of the remaining amino acids in the sequence is a preferred method for the chemical synthesis of the antigenic epitopes described herein. Techniques for solid phase synthesis are known to those skilled in the art.

Alternatively, the antigenic epitopes described herein are synthesized using recombinant nucleic acid methodology. Generally, this involves creating a nucleic acid sequence that encodes the peptide or protein, placing the nucleic acid in an expression cassette under the control of a particular promoter, expressing the peptide or protein in a host, isolating the expressed peptide or protein and, if required, renaturing the peptide or protein. Techniques sufficient to guide one of skill through such procedures are found in the literature.

When several desired protein fragments or peptides are encoded in the nucleotide sequence incorporated into a vector, one of skill in the art will appreciate that the protein fragments or peptides may be separated by a spacer molecule such as, for example, a peptide, consisting of one or more amino acids. Generally, the spacer will have no specific biological activity other than to join the desired protein fragments or peptides together, or to preserve some minimum distance or other spatial relationship between them. However, the constituent amino acids of the spacer may be selected to influence some property of the molecule such as the folding, net charge, or hydrophobicity. Nucleotide sequences encoding for the production of residues which may be useful in purification of the expressed recombinant protein may be built into the vector. Such sequences are

known in the art. For example, a nucleotide sequence encoding for a poly histidine sequence may be added to a vector to facilitate purification of the expressed recombinant protein on a nickel column.

Once expressed, recombinant peptides, polypeptides and proteins can be purified according to standard procedures known to one of ordinary skill in the art, including ammonium sulfate precipitation, affinity columns, column chromatography, gel electrophoresis and the like. Substantially pure compositions of about 50 to 99% homogeneity are preferred, and 80 to 95% or greater homogeneity are most preferred for use as therapeutic agents.

One of skill in the art will recognize that after chemical synthesis, biological expression or purification, the desired proteins, fragments thereof and peptides may possess a conformation substantially different than the native conformations of the proteins, fragments thereof and peptides. In this case, it is often necessary to denature and reduce protein and then to cause the protein to re-fold into the preferred conformation. Methods of reducing and denaturing proteins and inducing re-folding are well known to those of skill in the art.

The genetic constructs of the present invention include coding sequences for different proteins, fragments thereof, and peptides. The genetic constructs also include epitopes or domains chosen to permit purification or detection of the expressed protein. Such epitopes or domains include DNA sequences encoding the glutathione binding domain from glutathione S-transferase, hexa-histidine, thioredoxin, hemagglutinin antigen, maltose binding protein, and others commonly known to one of skill in the art. The preferred genetic construct includes the nucleotide sequences of SEQ ID NO: 1 and SEQ ID NO: 3 or fragments or conservative substitutions thereof. It is to be understood that additional or alternative nucleotide sequences may be included in the genetic constructs in order to encode for the following: a) multiple copies of the desired proteins, fragments thereof, or peptides; b) various combinations of the desired proteins, fragments thereof, or peptides; and c) conservative modifications of the desired proteins, fragments thereof, or peptides, and combinations thereof. Still another preferred

protein of the present invention is human duox2 (SEQ ID NO: 2) protein, and fragments or conservative substitutions thereof, as encoded by SEQ ID NO: 1 and fragments or conservative substitutions thereof. Another preferred protein of the present invention is Ce Duox 1 (SEQ ID NO:4) protein and fragments or conservative substitutions thereof, as encoded by SEQ ID NO: 3 and fragments or conservative substitutions thereof.The nucleotide sequences of the present invention may also be employed to hybridize to nucleic acids such as DNA or RNA nucleotide sequences under high stringency conditions which permit detection, for example, of alternately spliced messages.

The genetic construct is expressed in an expression system such as in NIH 3T3 cells using recombinant sequences in a pcDNA-3 vector (Invitrogen, Carlsbad, CA) to produce a recombinant protein. Preferred expression systems include but are not limited to Cos-7 cells, insect cells using recombinant baculovirus, and yeast. It is to be understood that other expression systems known to one of skill in the art may be used for expression of the genetic constructs of the present invention. A preferred protein of the present invention is referred to herein as human duox2, or fragments or conservative substitutions thereof, which has the amino acid sequence set forth in SEQ ID NO:2, or an amino acid sequence having amino acid substitutions as defined in the definitions that do not significantly alter the function of the recombinant protein in an adverse manner. Another preferred protein of the present invention is Ce Duox 1 (SEQ ID NO: 4) or fragments or conservative substitutions thereof, as encoded by SEQ ID NO: 3 and fragments or conservative substitutions thereof.

Terminology

As described herein, the term "human duox2" refers to a protein comprising an amino acid sequence as set forth in SEQ ID NO:2, or a fragment or conservative substitution thereof, and encoded by the nucleotide sequence as set forth in SEQ ID NO:1, or a fragment or conservative substitution thereof. Ce duox refers to duox from *C. elegans* or a fragment or conservative substitution thereof.

Construction of the Recombinant Gene

The desired gene is ligated into a transfer vector, such as pcDNA3, and the recombinants are used to transform host cells such as Cos-7 cells. It is to be understood that different transfer vectors, host cells, and transfection methods may be employed as commonly known to one of ordinary skill in the art. Two desired genes for use in transfection are shown in SEQ ID NO: 1, and SEQ ID NO: 3. For example, lipofectamine-mediated transfection and *in vivo* homologous recombination is used to introduce the duox1 gene into NIH 3T3 cells.

The synthetic gene is cloned and the recombinant construct containing duox gene is produced and grown in confluent monolayer cultures of a Cos-7 cell line. The expressed recombinant protein is then purified, preferably using affinity chromatography techniques, and its purity and specificity determined by known methods.

A variety of expression systems may be employed for expression of the recombinant protein. Such expression methods include, but are not limited to the following: bacterial expression systems, including those utilizing *E. coli* and *Bacillus subtilis*; virus systems; yeast expression systems; cultured insect and mammalian cells; and other expression systems known to one of ordinary skill in the art.

Transfection of Cells

It is to be understood that the vectors of the present invention may be transfected into any desired cell or cell line. Both *in vivo* and *in vitro* transfection of cells are contemplated as part of the present invention. Preferred cells for transfection include but are not limited to the following: fibroblasts (possibly to enhance wound healing and skin formation), granulocytes (possible benefit to increase function in a compromised immune system as seen in AIDS, and aplastic anemia), muscle cells, neuroblasts, stem cells, bone marrow cells, osteoblasts, B lymphocytes, and T lymphocytes.

Cells may be transfected with a variety of methods known to one of ordinary skill in the art and include but are not limited to the following: electroporation, gene gun, calcium phosphate, lipofectamine, and fugene, as well as adenoviral transfection systems.

Host cells transfected with the nucleic acids represented in SEQ ID NO: 1, and SEQ ID NO: 3, or fragments or conservative substitutions thereof, are used to express the proteins SEQ ID NO: 2 and SEQ ID NO: 4, respectively, or fragments or conservative substitutions thereof.

These expressed proteins are used to raise antibodies. These antibodies may be used for a variety of applications including but not limited to immunotherapy against cancers expressing one of the duox proteins, for affecting cuticle formation, and for detection, localization and measurement of the proteins shown in SEQ ID NO: 2 and SEQ ID NO: 4, or fragments or conservative substitutions thereof.

Purification and Characterization of the Expressed Protein

The proteins of the present invention can be expressed as a fusion protein with a poly histidine component, such as a hexa histidine, and purified by binding to a metal affinity column using nickel or cobalt affinity matrices. The protein can also be expressed as a fusion protein with glutathione S-transferase and purified by affinity chromatography using a glutathione agarose matrix. The protein can also be purified by immunoaffinity chromatography by expressing it as a fusion protein, for example with hemagglutinin antigen. The expressed or naturally occurring protein can also be purified by conventional chromatographic and purification methods which include anion and cation exchange chromatography, gel exclusion chromatography, hydroxylapatite chromatography, dye binding chromatography, ammonium sulfate precipitation, precipitation in organic solvents or other techniques commonly known to one of skill in the art.

Methods of Assessing Activity of Expressed Proteins

Different methods are available for assessing the activity of the expressed proteins of the present invention, including, but not limited to, the proteins represented as SEQ ID NO: 2 and SEQ ID NO: 4, substituted analogs thereof, and fragments or conservative substitutions thereof.

1. Assays of the holoprotein and fragments thereof for superoxide generation:

A. General considerations.

These assays are useful in assessing efficacy of drugs designed to modulate the activity of the enzymes of the present invention. The holoprotein may be expressed in COS-7 cells, NIH 3T3 cells, insect cells (using baculoviral technology) or other cells using methods known to one of skill in the art. Membrane fractions or purified protein are used for the assay. The assay may require or be augmented by other cellular proteins such as p47phox, p67phox, and Rac1, as well as potentially other unidentified factors (e.g., kinases or other regulatory proteins).

B. Cytochrome c reduction.

NADPH or NADH is used as the reducing substrate, in a concentration of about 100 μM. Reduction of cytochrome c is monitored spectrophotometrically by the increase in absorbance at 550 nm, assuming an extinction coefficient of 21 $mM^{-1}cm^{-1}$. The assay is performed in the absence and presence of about 10 μg superoxide dismutase. The superoxide-dependent reduction is defined as cytochrome c reduction in the absence of superoxide dismutase minus that in the presence of superoxide dismutase (Uhlinger et al. (1991) *J. Biol. Chem.* 266, 20990-20997). Acetylated cytochrome c may also be used, since the reduction of acetylated cytochrome c is thought to be exclusively via superoxide.

C. Nitroblue tetrazolium reduction.

For nitroblue tetrazolium (NBT) reduction, the same general protocol is used, except that NBT is used in place of

cytochrome c. In general, about 1 mL of filtered 0.25 % nitrotetrazolium blue (Sigma, St. Louis, MO) is added in Hanks buffer without or with about 600 Units of superoxide dismutase (Sigma) and samples are incubated at approximately 37°C. The oxidized NBT is clear, while the reduced NBT is blue and insoluble. The insoluble product is collected by centrifugation, and the pellet is re-suspended in about 1 mL of pyridine (Sigma) and heated for about 10 minutes at 100°C to solubilize the reduced NBT. The concentration of reduced NBT is determined by measuring the absorbance at 510 nm, using an extinction coefficient of 11,000 $M^{-1}cm^{-1}$. Untreated wells are used to determine cell number.

D. Luminescence.

Superoxide generation may also be monitored with a chemiluminescence detection system utilizing lucigenin (bis-N-methylacridinium nitrate, Sigma, St. Louis, MO). The sample is mixed with about 100 μM NADPH (Sigma, St. Louis, MO) and 10 μM lucigenin (Sigma, St. Louis, MO) in a volume of about 150 μL Hanks solution. Luminescence is monitored in a 96-well plate using a LumiCounter (Packard, Downers Grove, IL) for 0.5 second per reading at approximately 1 minute intervals for a total of about 5 minutes; the highest stable value in each data set is used for comparisons. As above, superoxide dismutase is added to some samples to prove that the luminescence arises from superoxide. A buffer blank is subtracted from each reading (Ushio-Fukai et al. (1996) *J. Biol. Chem.* 271, 23317-23321).

E. Assays in intact cells.

Assays for superoxide generation may be performed using intact cells, for example, the duox-transfected NIH 3T3 cells. In principle, any of the above assays can be used to evaluate superoxide generation using intact cells, for example, the duox-transfected NIH 3T3 cells. NBT reduction is a preferred assay method.

2. Assays of truncated proteins comprised of approximately the C-terminal 265 amino acid residues

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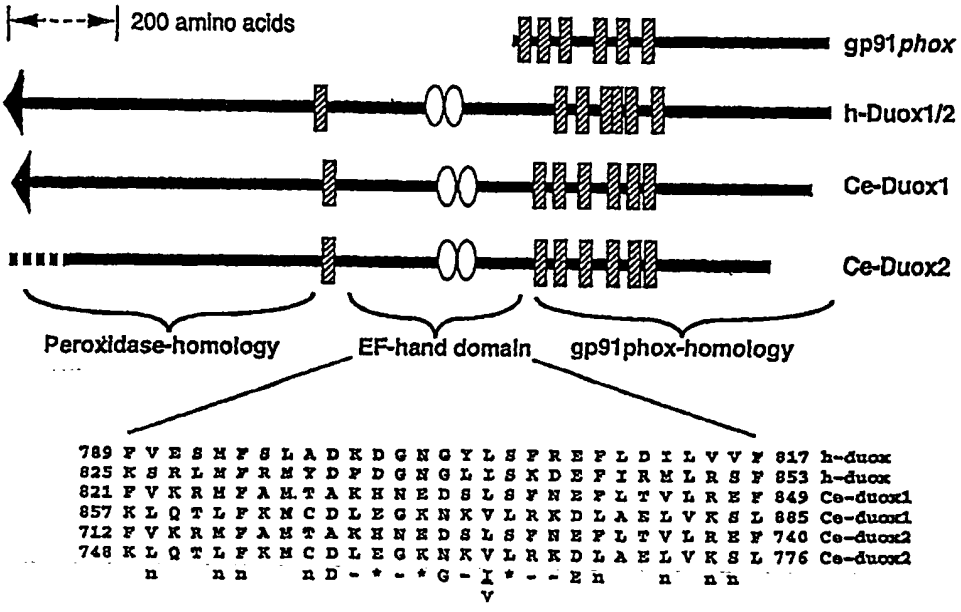
(54) Title: NOVEL DUAL OXIDASES AS MITOGENIC AND ENDOCRINE REGULATORS



(57) Abstract: The present invention relates to new genes encoding for the production of novel proteins involved in generation of reactive oxygen intermediates and in peroxidative reactions that affect biological functions including cell division, thyroid hormone biosynthesis and tissue fibrosis. The present invention also provides vectors containing these genes, cells transfected with these vectors, antibodies raised against these novel proteins, kits for detection, localization and measurement of these genes and proteins, and methods to determine the activity of drugs to affect the activity of the proteins of the present invention.

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NOVEL DUAL OXIDASES AS MITOGENIC AND ENDOCRINE REGULATORS

The U.S. Government has a paid-up license in this invention and the right in limited circumstances to require the patent owner to license others on reasonable terms as provided for by the terms of National Institutes of Health grants HL38206, HL58000 and CA84138.

TECHNICAL FIELD

The present invention relates to the fields of normal and abnormal cell growth, in particular mitogenic regulation, to thyroid hormone biosynthesis and to nematode cuticle biogenesis. The present invention provides the following: nucleotide sequences encoding for the production of enzymes that are mitogenic regulators, that catalyze thyroid hormone biosynthesis and in nematodes catalyze the biogenesis of cuticle; amino acid sequences of these enzymes; vectors containing these nucleotide sequences; methods for transfecting cells with vectors that produce these enzymes; and antibodies to these enzymes that are useful for detecting and measuring levels of these enzymes, and for binding to cells possessing extracellular epitopes of these enzymes.

BACKGROUND OF THE INVENTION

Reactive oxygen intermediates (ROI) are partial reduction products of oxygen: 1 electron reduces O_2 to form superoxide (O_2^-), and 2 electrons reduce O_2 to form hydrogen peroxide (H_2O_2). ROI are generated as a byproduct of aerobic metabolism and by toxicological mechanisms. There is growing evidence for regulated enzymatic generation of O_2^- and its conversion

to H_2O_2 in a variety of cells. The conversion of O_2^- to H_2O_2 occurs spontaneously, but is markedly accelerated by superoxide dismutase (SOD). High levels of ROI are associated with damage to biomolecules such as DNA, biomembranes and proteins. Recent evidence indicates generation of ROI under normal cellular conditions and points to signaling and metabolic roles for O_2^- and H_2O_2.

Several biological systems generate reactive oxygen. Phagocytic cells such as neutrophils generate large quantities of ROI as part of their battery of bactericidal mechanisms. Exposure of neutrophils to bacteria or to various soluble mediators such as formyl-Met-Leu-Phe or phorbol esters activates a massive consumption of oxygen, termed the respiratory burst, to initially generate superoxide, with secondary generation of H_2O_2, HOCl and hydroxyl radical. The enzyme responsible for this oxygen consumption is the respiratory burst oxidase (nicotinamide adenine dinucleotide phosphate-reduced form (NADPH) oxidase).

There is growing evidence for the generation of ROI by non-phagocytic cells, particularly in situations related to cell proliferation. In addition, in some tissues such as thyroid, ROI generation is implicated in metabolic conversions such as the biosynthesis of thyroid hormone. Significant generation of H_2O_2, O_2^-, or both have been noted in some cell types. Fibroblasts and human endothelial cells show increased release of superoxide in response to cytokines such as interleukin-1 or tumor necrosis factor (TNF) (Meier et al. (1989) *Biochem J.* 263, 539-545.; Matsubara et al. (1986) *J. Immun.* 137, 3295-3298). Ras-transformed fibroblasts show increased superoxide release compared with control fibroblasts (Irani, et al. (1997) *Science* 275, 1649-1652). Rat vascular smooth muscle cells show increased H_2O_2 release in response to PDGF (Sundaresan et al. (1995) *Science* 270, 296-299) and angiotensin II (Griendling et al. (1994) *Circ. Res.* 74, 1141-1148; Fukui et al. (1997) *Circ. Res.* 80, 45-51; Ushio-Fukai et al. (1996) *J. Biol. Chem.* 271, 23317-23321), and H_2O_2 in these cells is associated with increased proliferation rate. The occurrence of ROI in a variety of cell types is summarized in Table 1 (adapted from Burdon, R. (1995) *Free Radical Biol. Med.* 18, 775-794).

Table 1

Superoxide	Hydrogen Peroxide
human fibroblasts	Balb/3T3 cells
human endothelial cells	rat pancreatic isletcells
human/rat smooth muscle cells	murine keratinocytes
human fat cells	rabbit chondrocytes
human osteocytes	human tumor cells
BHK-21 cells	fat cells, 3T3 L1 cells
human colonic epithelial cells	

ROI generated by the neutrophil have a cytotoxic function. While ROI are normally directed at the invading microbe, ROI can also induce tissue damage (e.g., in inflammatory conditions such as arthritis, shock, lung disease, and inflammatory bowel disease) or may be involved in tumor initiation or promotion, due to damaging effects on DNA. Nathan (Szatrowski et al. (1991) *Canc. Res.* 51, 794-798) proposed that the generation of ROI in tumor cells may contribute to the hypermutability seen in tumors, and may therefore contribute to tumor heterogeneity, invasion and metastasis.

In addition to cytotoxic and mutagenic roles, ROI have ideal properties as signal molecules: 1) they are generated in a controlled manner in response to upstream signals; 2) the signal can be terminated by rapid metabolism of O_2^- and H_2O_2 by SOD and catalase/peroxidases; 3) they elicit downstream effects on target molecules, e.g., redox-sensitive regulatory proteins such as NF kappa B and AP-1 (Schreck et al. (1991) *EMBO J.* 10, 2247-2258; Schmidt et al. (1995) *Chemistry & Biology* 2, 13-22). Oxidants such as O_2^- and H_2O_2 have a relatively well defined signaling role in bacteria, operating via the SoxI/II regulon to regulate transcription.

ROI appear to have a direct role in regulating cell division, and may function as mitogenic signals in pathological conditions related to growth. These conditions include cancer and cardiovascular disease. O_2^- is generated in endothelial cells in response to cytokines, and might play a role in angiogenesis (Matsubara et al. (1986) *J. Immun.* 137, 3295-3298). O_2^- and H_2O_2 are also proposed to function as "life-signals", preventing cells from undergoing apoptosis (Matsubara et al. (1986) *J. Immun.* 137, 3295-3298). As discussed above, many cells respond to growth factors (e.g., platelet derived growth factor (PDGF), epidermal derived growth factor (EGF), angiotensin II, and various cytokines) with both increased production of O_2^- /H_2O_2 and increased proliferation. Inhibition of ROI generation prevents the mitogenic response. Exposure to exogenously generated O_2^- and H_2O_2 results in an increase in cell proliferation. A partial list of responsive cell types is shown below in Table 2 (adapted from Burdon, R. (1995) *Free Radical Biol. Med.* 18, 775-794).

Table 2

Superoxide	Hydrogen peroxide
human, hamster fibroblasts	mouse osteoblastic cells
Balb/3T3 cells	Balb/3T3 cells
human histiocytic leukemia	rat, hamster fibroblasts
mouse epidermal cells	human smooth muscle cells
rat colonic epithelial cells	rat vascular smooth muscle
rat vascular smooth muscle cells	cells

While non-transformed cells can respond to growth factors and cytokines with the production of ROI, tumor cells appear to produce ROI in an uncontrolled manner. A series of human tumor cells produced large amounts of hydrogen peroxide compared with non-tumor cells (Szatrowski et al. (1991) *Canc. Res.* 51, 794-798). Ras-transformed NIH 3T3 cells generated elevated amounts of

superoxide, and inhibition of superoxide generation by several mechanisms resulted in a reversion to a "normal" growth phenotype.

O_2^- has been implicated in maintenance of the transformed phenotype in cancer cells including melanoma, breast carcinoma, fibrosarcoma, and virally transformed tumor cells. Decreased levels of the manganese form of SOD (MnSOD) have been measured in cancer cells and *in vitro*-transformed cell lines, predicting increased O_2^- levels (Burdon, R. (1995) *Free Radical Biol. Med.* 18, 775-794). MnSOD is encoded on chromosome 6q25 which is very often lost in melanoma. Overexpression of MnSOD in melanoma and other cancer cells (Church et al. (1993) *Proc. of Natl. Acad. Sci.* 90, 3113-3117; Fernandez-Pol et al. (1982) *Canc. Res.* 42, 609-617; Yan et al. (1996) *Canc. Res.* 56, 2864-2871) resulted in suppression of the transformed phenotype.

ROI are implicated in growth of vascular smooth muscle associated with hypertension, atherosclerosis, and restenosis after angioplasty. O_2^- generation is seen in rabbit aortic adventitia (Pagano et al. (1997) *Proc. Natl. Acad. Sci.* 94, 14483-14488). Vascular endothelial cells release O_2^- in response to cytokines (Matsubara et al. (1986) *J. Immun.* 137, 3295-3298). O_2^- is generated by aortic smooth muscle cells in culture, and increased O_2^- generation is stimulated by angiotensin II which also induces cell hypertrophy. In a rat model system, infusion of angiotensin II leads to hypertension as well as increased O_2^- generation in subsequently isolated aortic tissue (Ushio-Fukai et al. (1996) *J. Biol. Chem.* 271, 23317-23321.; Yu et al. (1997) *J. Biol. Chem.* 272, 27288-27294). Intravenous infusion of a form of SOD that localizes to the vasculature or an infusion of an O_2^- scavenger prevented angiotensin II induced hypertension and inhibited ROI generation (Fukui et al. (1997) *Circ. Res.* 80, 45-51).

The neutrophil NADPH oxidase, also known as phagocyte respiratory burst oxidase, provides a paradigm for the study of the specialized enzymatic ROI-generating system. This extensively studied enzyme oxidizes NADPH and reduces oxygen to form O_2^-. NADPH oxidase consists of multiple proteins and is regulated by assembly of cytosolic and membrane components. The catalytic

moiety consists of flavocytochrome b_{558}, an integral plasma membrane enzyme comprised of two components: gp91phox (gp refers to glycoprotein; phox is an abbreviation of the words phagocyte and oxidase) and p22phox (p refers to protein). gp91phox contains 1 flavin adenine dinucleotide (FAD) and 2 hemes as well as the NADPH binding site.

p22phox has a C-terminal proline-rich sequence which serves as a binding site for cytosolic regulatory proteins. The two cytochrome subunits, gp91phox and p22phox appear to stabilize one another, since the genetic absence of either subunit, as in the inherited disorder chronic granulomatous disease (CGD), results in the absence of the partner subunit (Yu et al. (1997) *J. Biol. Chem.* 272, 27288-27294). Essential cytosolic proteins include p47phox, p67phox and the small GTPase Rac, of which there are two isoforms. p47phox and p67phox both contain SH_3 regions and proline-rich regions which participate in protein interactions governing assembly of the oxidase components during activation. The neutrophil enzyme is regulated in response to bacterial phagocytosis or chemotactic signals by phosphorylation of p47phox, and perhaps other components, as well as by guanine nucleotide exchange to activate the GTP-binding protein Rac.

The origin of ROI in non-phagocytic tissues is unproven, but the occurrence of phagocyte oxidase components has been evaluated in several systems by immunochemical methods, Northern blots and reverse transcriptase-polymerase chain reaction (RT-PCR). The message for p22phox is expressed widely, as is that for Rac1. Several cell types that are capable of O_2^- generation have been demonstrated to contain all of the phox components including gp91phox, as summarized below in Table 3. These cell types include endothelial cells, aortic adventitia and lymphocytes.

Table 3

Tissue	gp91phox	p22phox	p47phox	p67phox
neutrophil	$+^{1,2}$	$+^{1,2}$	$+^{1,2}$	$+^{1,2}$
aortic adventitia	$+^{1}$	$+^{1}$	$+^{1}$	$+^{1}$
lymphocytes	$+^{2}$	$+^{2}$	$+^{1,2}$	$+^{1,2}$
endothelial cells	$+^{2}$	$+^{2}$	$+^{1,2}$	$+^{1,2}$
glomerular mesangial cells	-	$+^{1,2}$	$+^{1,2}$	$+^{1,2}$
fibroblasts	-	$+^{2}$	$+^{1,2}$	$+^{2}$
aortic sm. muscle	-	$+^{1,2}$	?	?

1= protein expression shown. 2= mRNA expression shown.

A distinctly different pattern is seen in several other cell types shown in Table 3 including glomerular mesangial cells, rat aortic smooth muscle and fibroblasts. In these cells, expression of gp91phox is absent while p22phox and in some cases cytosolic phox components have been demonstrated to be present. Since gp91phox and p22phox stabilize one another in the neutrophil, there has been much speculation that some molecule, possibly related to gp91phox, accounts for ROI generation in glomerular mesangial cells, rat aortic smooth muscle and fibroblasts (Ushio-Fukai et al. (1996) *J. Biol. Chem.* 271, 23317-23321). Investigation of fibroblasts from a patient with a genetic absence of gp91phox provides proof that the gp91phox subunit is not involved in ROI generation in these cells (Emmendorffer et al. (1993) *Eur. J. Haematol.* 51, 223-227). Depletion of p22phox from vascular smooth muscle using an antisense approach indicated that this subunit participates in ROI generation in these cells, despite the absence of detectable gp91phox (Ushio-Fukai et al. (1996) *J. Biol. Chem.* 271, 23317-23321).

Thyroid hormone regulates basal metabolic rate through end-effects on mitochondrial respiration, and conditions of under- or over-production are important clinically. Development of drugs to regulate the biosynthesis of thyroid hormone is a medically important goal, and identification of the enzymes in this pathway is key to developing

pharmacologically relevant targets. Thyroid uniquely concentrates iodide, which is used to iodinate tyrosine residues on thyroglobulin (TG). TG is a large protein (660 kDa) that contains 67 tyrosyl residues, some of which are preferential sites for iodination. Iodination of tyrosines in TG is catalyzed by thyroid peroxidase (TPO), a plasma membrane hemoprotein. Iodination requires a previously unidentified enzymatic source of H_2O_2. A second step is the coupling of two diiodotyrosines (DIT) to form protein-associated thyroxine (T4), which is subsequently proteolytically cleaved from TG to liberate free T4. What is needed is a composition of the gene that encodes the enzyme that generates H_2O_2 in thyroid and that catalyzes the coupling reaction, and a method of using that composition to modulate thyroid hormone biosynthesis. Such information would be useful in the development of drugs for modulation of thyroid function. Such modulation might be useful in the treatment of hyperthyroidism.

Recent evidence suggests that enzymes involved in oxidative cross-linking of tyrosine in growth factor stimulated fibroblasts may lead to fibrotic damage. Lung fibrosis is particularly damaging to individuals afflicted with this condition. Identification of the genes encoding enzymes involved in such oxidative cross-linking reactions is needed so that drugs may be designed to alleviate or prevent fibrotic damage, particularly in the lung.

Parasitic diseases are a major cause of morbidity and mortality worldwide in humans and animals, and have a significant impact on agricultural productivity as well. Parasitic diseases have proven difficult to treat, in part due to the presence of the cuticle, a tough exoskeletal structure of parasites such as nematodes. What is needed is a composition and method of using the composition to fight parasitic diseases, including but not limited to those parasitic diseases caused by parasites with cuticles.

Accordingly, what is needed is a method of disrupting the formation of the cuticle which would make the worm susceptible to the host defense mechanisms and drug treatment.

What is also needed is the identity of the proteins involved in ROI generation, especially in non-phagocytic tissues and

cells. What is also needed are the nucleotide sequences encoding for these proteins, and the primary sequences of the proteins themselves. Also needed are vectors designed to include nucleotides encoding for these proteins. Probes and PCR primers derived from the nucleotide sequence are needed to detect, localize and measure nucleotide sequences, including mRNA, involved in the synthesis of these proteins. In addition, what is needed is a means to transfect cells with these vectors. What is also needed are expression systems for production of these molecules. Also needed are antibodies directed against these molecules for a variety of uses including localization, detection, and measurement and passive immunization.

SUMMARY OF THE INVENTION

The present invention solves the problems described above by providing a novel family of nucleotide sequences and proteins encoded by these nucleotide sequences termed duox proteins. In particular, the present invention provides compositions comprising the nucleotide sequences SEQ ID NO: 1 and SEQ ID NO: 3, and fragments and conservative substitutions thereof, which encode for the expression of proteins comprising SEQ ID NO: 2 and SEQ ID NO: 4 respectively, and fragments and conservative substitutions thereof. Preferred protein fragments include, but are not limited to, SEQ ID NO: 31 and SEQ ID NO: 32. While not wanting to be bound by the following statement, it is believed that these proteins are involved in ROI production and are capable of stimulating superoxide production or generating peroxidative reactions. The present invention also provides vectors containing these nucleotide sequences, cells transfected with these vectors which produce the proteins comprising SEQ ID NO: 2 and SEQ ID NO: 4, and fragments and conservative substitutions thereof, and antibodies to these proteins and fragments and conservative substitutions thereof. The present invention also provides methods for stimulating cellular proliferation by administering vectors encoded for production of the proteins comprising SEQ ID NO: 2 and SEQ ID NO: 4 and fragments and conservative substitutions thereof. The present invention also provides methods for stimulating cellular proliferation by

administering the proteins comprising SEQ ID NO: 2 and SEQ ID NO: 4 and fragments and conservative substitutions thereof. The proteins comprising SEQ ID NO: 2 and SEQ ID NO: 4 and fragments and conservative substitutions thereof are useful in affecting the exoskeleton, especially the cuticle of parasites, including but not limited to nematodes. The nucleotides and antibodies of the present invention are useful for the detection, localization and measurement of the nucleic acids encoding for the production of the proteins of the present invention, and also for the detection, localization and measurement of the proteins of the present invention. These nucleotides and antibodies can be combined with other reagents in kits for the purposes of detection, localization and measurement.

Most particularly, the present invention involves a method for regulation of cell division or cell proliferation by modifying the activity or expression of the duox proteins described as SEQ ID NO: 2 and SEQ ID NO: 4 or fragments or conservative substitutions thereof. These proteins, in their naturally occurring or expressed forms, are expected to be useful in drug development, for example for screening of chemical and drug libraries by observing inhibition of the activity of these enzymes. Such chemicals and drugs would likely be useful as treatments for cancer, prostatic hypertrophy, benign prostatic hypertrophy, hypertension, metabolic disease, fibrosis, atherosclerosis and many other disorders involving abnormal cell growth or proliferation, and a variety of parasitic diseases in both animals and crops as described below. The entire expressed protein may be useful in these assays. Portions of the molecule which may be targets for inhibition or modification include but are not limited to the binding site for pyridine nucleotides (NADPH or NADH), the flavoprotein domain (approximately the C-terminal 265 amino acids), and/or the binding or catalytic site for flavin adenine dinucleotide (FAD).

The method of the present invention may be used for the development of drugs or other therapies for the treatment of conditions associated with abnormal growth including, but not limited to the following: cancer, fibrosis, lung fibrosis, metabolic imbalances, thyroid imbalances, hyperthryoidism, psoriasis, prostatic hypertrophy,

benign prostatic hypertrophy, cardiovascular disease, proliferation of vessels, including but not limited to blood vessels and lymphatic vessels, arteriovenous malformation, vascular problems associated with the eye, atherosclerosis, hypertension, and restenosis following angioplasty and parasitic diseases. The enzymes of the present invention are excellent targets for the development of drugs and other agents which may modulate the activity of these enzymes. It is to be understood that modulation of activity may result in enhanced, diminished or absence of enzymatic activity. Modulation of the activity of these enzymes may be useful in treatment of conditions, including but not limited to conditions associated with abnormal growth, metabolic disorders, and fibrosis.

Drugs which affect the activity of the duox enzymes represented in SEQ ID NO: 2 and SEQ ID NO: 4, or fragments or conservative substitutions thereof, may also be combined with other therapeutics in the treatment of specific conditions. For example, these drugs may be combined with angiogenesis inhibitors in the treatment of cancer, with antihypertensives for the treatment of hypertension, with cholesterol lowering drugs for the treatment of atherosclerosis and with hormonal agonists or antagonists in the treatment of endocrine disorders, such as thyroid disorders.

It is to be understood that the proteins of the present invention, including but not limited to, SEQ ID NO: 2 and SEQ ID NO: 4, or fragments or conservative substitutions thereof, may be administered together with other compositions such as anti-parasitic compositions, pesticides, herbicides and fertilizers. Accordingly, the proteins of the present invention may be useful alone or in combination with other compositions for treating humans or animals, including livestock, other farm animals and domestic animals, including pets, for preventing or fighting parasitic disease, for protecting plants and crops against attack by parasites, especially soil nematodes, and for destroying parasites.

Accordingly, an object of the present invention is to provide nucleotide sequences, or fragments thereof or conservative substitutions thereof, encoding for the production of proteins, or fragments thereof or conservative substitutions thereof, that are

involved in ROI production, stimulate superoxide production or generate peroxidative reactions.

It is another object of the present invention is to provide the proteins represented in SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 31, and SEQ ID NO: 32 or fragments or conservative substitutions thereof.

It is another object of the present invention is to provide the nucleotide sequences encoding for the proteins represented in SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 31, and SEQ ID NO: 32 or fragments or conservative substitutions thereof, wherein these nucleotide sequence include SEQ ID NO: 1, SEQ ID NO: 3 or fragments or conservative substitutions thereof.

It is another object of the present invention to provide proteins, fragments thereof or conservative substitutions thereof, involved in exoskeletal or cuticle formation that may be used as targets for therapies designed to prevent exoskeletal or cuticle formation and to harm organisms having an exoskeleton or cuticle, particularly parasites.

It is another object of the present invention to provide proteins, fragments thereof or conservative substitutions thereof, involved in thyroid hormone biosynthesis that may be used as targets for therapies designed to inhibit biosynthesis of thyroid hormone.

It is yet another object of the present invention to provide proteins, fragments thereof or conservative substitutions thereof, involved in tissue fibrosis that may be used as targets for therapies designed to prevent fibrosis.

Another object of the present invention is to provide proteins involved in lung fibrosis that may be used as targets for therapies designed to prevent lung fibrosis.

Another object of the present invention is to provide vectors containing these nucleotide sequences, or fragments thereof.

Yet another object of the present invention is to provide cells transfected with these vectors.

Still another object of the present invention is to administer cells transfected with these vectors to animals and humans.

Another object of the present invention is to provide proteins, or fragments thereof, that are involved in ROI production, stimulate superoxide production or generate peroxidative reactions.

Still another object of the present invention is to provide antibodies, including monoclonal and polyclonal antibodies, or fragments thereof, raised against proteins, or fragments thereof or conservative substitutions thereof, that are involved in ROI production, stimulate superoxide production or generate peroxidative reactions. Such antibodies are useful in the localization and measurement of proteins, or fragments thereof, that are involved in ROI production.

Another object of the present invention is to administer genes containing nucleotide sequences, or fragments thereof, encoding for the production of proteins, or fragments thereof or conservative substitutions thereof, that are involved in ROI production, stimulate superoxide production or generate peroxidative reactions, to animals and humans and also to cells obtained from animals and humans.

Another object of the present invention is to administer antisense complimentary sequences of genes containing nucleotide sequences, or fragments thereof or conservative substitutions thereof, encoding for the production of proteins, or fragments thereof or conservative substitutions thereof, that are involved in ROI production, stimulate superoxide production or generate peroxidative reactions, to animals and humans and also to cells obtained from animals and humans.

Yet another object of the present invention is to provide a method for stimulating or inhibiting cellular proliferation by administering vectors containing nucleotide sequences, or fragments thereof, encoding for the production of proteins, or fragments thereof, that are involved in ROI production, stimulate superoxide production or generate peroxidative reactions, to animals and humans. It is also an object of the present invention to provide a method for stimulating or inhibiting cellular proliferation by administering vectors containing antisense complimentary sequences of nucleotide sequences, or fragments thereof, encoding for the production of proteins, or fragments thereof, that are involved in ROI production, stimulate

superoxide production or generate peroxidative reactions, to animals and humans. These methods of stimulating cellular proliferation are useful for a variety of purposes, including but not limited to, developing animal models of tumor formation, stimulating cellular proliferation of blood marrow cells following chemotherapy or radiation, or in cases of anemia.

Yet another object of the present invention is to provide nucleotide probes useful for the detection, localization and measurement of nucleotide sequences, or fragments thereof, encoding for the production of proteins, or fragments thereof, that are involved in ROI production, stimulate superoxide production or generate peroxidative reactions.

Another object of the present invention is to provide kits useful for detection of nucleic acids including the nucleic acids represented in SEQ ID NO: 1, and SEQ ID NO: 3, or fragments thereof or conservative substitutions thereof, that encode for proteins, or fragments thereof or conservative substitutions thereof, that are involved in ROI production, stimulate superoxide production or generate peroxidative reactions.

Yet another object of the present invention is to provide kits useful for detection and measurement of nucleic acids including the nucleic acids represented in SEQ ID NO: 1, and SEQ ID NO: 3, or fragments thereof, that encode for proteins, or fragments thereof or conservative substitutions thereof, that are involved in ROI production, stimulate superoxide production or generate peroxidative reactions.

Another object of the present invention is to provide kits useful for detection of proteins, including the proteins represented in SEQ ID NO: 2 and SEQ ID NO: 4 or fragments thereof, that are involved in ROI production, stimulate superoxide production or generate peroxidative reactions.

Yet another object of the present invention is to provide kits useful for detection and measurement of proteins, including the proteins represented in SEQ ID NO: 2 and SEQ ID NO: 4, or fragments thereof, that are involved in ROI production, stimulate superoxide production or generate peroxidative reactions.

Still another object of the present invention is to provide kits useful for localization of proteins, including the proteins represented in SEQ ID NO: 2 and SEQ ID NO: 4, or fragments thereof, that are involved in ROI production, stimulate superoxide production or generate peroxidative reactions.

Yet another object of the present invention is to provides kits useful for the detection, measurement or localization of nucleic acids, or fragments thereof, encoding for proteins, or fragments thereof, that are involved in ROI production, for use in diagnosis and prognosis of abnormal cellular proliferation related to ROI production.

Another object of the present invention is to provides kits useful for the detection, measurement or localization of proteins, or fragments thereof, that are involved in ROI production, for use in diagnosis and prognosis of abnormal cellular proliferation related to ROI production.

These and other objects, features and advantages of the present invention will become apparent after a review of the following detailed description of the disclosed embodiments and the appended drawings.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Structure of large homologs of gp91*phox*. Domain structure of Duox proteins. Secretory signal peptide sequences are indicated by a gray triangle, while predicted transmembrane alpha helices are indicated by a hashed rectangle. White ovals indicate regions showing homology with EF-hand calcium binding sites.

Figure 2. Comparison of the peroxidase domains of h-Duox, Ce-Duox1 and some known peroxidases.

A) Sequence alignments. Abbreviations are: MPO, myeloperoxidase; TPO, thyroid peroxidase; EPO, eosinophil peroxidase; LPO, lactoperoxidase, Pxsn.dros, *Drosophila* peroxidasin. Residues which are conserved among all 7 proteins are shown with black boxes, while those matching a derived consensus sequence are shown in line boxes. Filled circles indicate residues which are

proposed to provide contacts with the heme, based on the crystal structure of canine myeloperoxidase (Zeng and Fenna, 1992). The superscripted double bar indicates residues comprising a calcium binding region, and filled triangles indicate residues which appear in the crystal structure to bind directly to the calcium ion.

B) Phylogenetic relationships. The sequences shown in A as well as additional sequences are shown. Abbreviations are: OPO, ovoperoxidase; str.purp, *Strongylocentrotus purpuratus*; ly.var, *Lytechinus variegatus*; hemi.pulch; *Hemicentrotus pulcherrimus*.

Figure 3. Tissue expression of mRNA for h-Duox. mRNA for h-Duox1, h-Duox2 and glyceraldehyde 3-phosphate dehydrogenase was detected by RT-PCR.

DETAILED DESCRIPTION OF THE INVENTION

The present invention solves the problems described above by providing a novel family of nucleotide sequences and proteins, encoded by these nucleotide sequences, termed duox proteins. The term "duox" refers to "dual oxidase". In particular, the present invention provides novel compositions comprising the nucleotide sequences SEQ ID NO: 1, and SEQ ID NO: 3, and fragments thereof or conservative substitutions thereof, which encode, respectively, for the expression of proteins comprising SEQ ID NO: 2 and SEQ ID NO: 4 and fragments thereof or conservative substitutions thereof. Preferred protein fragments include, but are not limited to SEQ ID NO: 31 and SEQ ID NO: 32.

The duox proteins described herein have homology to the gp91phox protein involved in ROI generation, however, the duox proteins comprise a novel and distinct family of proteins. The duox proteins described herein have three distinct regions: the amino terminal region having homology to peroxidase proteins, the internal region having homology to calmodulin (CAM) proteins and the carboxy-terminal region having homology to mox (also called nox) proteins. The amino acid sequence of human duox2 is shown in SEQ ID NO: 2. Nucleotides encoding duox2 proteins are also shown in

SEQ ID NO: 1. In addition to the human duox proteins, comparison of the sequence of human duox1 and human duox2 with genomic databases using BLAST searching resulted in the identification of two homologs of duox in C. elegans (Ce-duox1 SEQ ID NO: 3) and the pseudogene Ce-duox2. Drosophila also appears to have at least one duox homolog. Thus, the duox family of genes/proteins is widely distributed among multicellular organisms.

High molecular weight homologs of gp91*phox*, have been identified in human (h) and *C. elegans* (Ce), and are termed Duox for "dual oxidase" because they have both a peroxidase-homology domain and a gp91*phox* domain. Ce-Duox uses cytosolic NADPH to generate reactive oxygen. It catalyzes cross-linking of free tyrosine ethyl ester involved in the stabilization of the cuticular extracellular matrix in nematodes.

Although not wanting to be bound by the following statement, it is believed that duox enzymes, for example duox2 and Ce-duox1, have dual enzymatic functions, catalyzing both the generation of superoxide and peroxidative type reactions. The latter class of reactions utilize hydrogen peroxide as a substrate (and in some cases have been proposed to utilize superoxide as a substrate). Since hydrogen peroxide is generated spontaneously from the dismutation of superoxide, it is believed that the NAD(P)H oxidase domain generates the superoxide and/or hydrogen peroxide which can then be used as a substrate for the peroxidase domain. In support of this hypothesis, a model for the duox2 protein in *C. elegans* has been developed that has an extracellular N-terminal peroxidase domain, a transmembrane region and a NADPH binding site located on the cytosolic face of the plasma membrane. By analogy with the neutrophil NADPH-oxidase which generates extracellular superoxide, human duox2 is predicted to generate superoxide and its byproduct hydrogen peroxide extracellularly where it can be utilized by the peroxidase domain.

The peroxidase domain is likely to confer additional biological functions. Depending upon the co-substrate, peroxidases can participate in a variety of reactions including halogenation such as the generation of hypochlorous acid (HOCl) by myeloperoxidase and

the iodination of tyrosine to form thyroxin by thyroid peroxidase. Peroxidases have also been documented to participate in the metabolism of polyunsaturated fatty acids, and in the chemical modification of tyrosine in collagen (by sea urchin ovoperoxidase). Although not wanting to be bound by this statement, it is believed that the predicted transmembrane nature of duox2 facilitates its function in the formation or modification of extracellular matrix or basement membrane. Since the extracellular matrix plays an important role in tumor cell growth, invasion and metastasis, it is believed that the duox type enzymes play a pathogenic role in such conditions.

In addition to the nucleotide sequences described above, the present invention also provides vectors containing these nucleotide sequences and fragments thereof or conservative substitutions thereof, cells transfected with these vectors which produce the proteins comprising SEQ ID NO: 2 and SEQ ID NO: 4 and fragments thereof or conservative substitutions thereof, and antibodies to these proteins and fragments thereof. The present invention also provides methods for stimulating cellular proliferation by administering vectors, or cells containing vectors, encoded for production of the proteins comprising SEQ ID NO: 2, SEQ ID NO: 4, and fragments thereof. The nucleotides and antibodies of the present invention are useful for the detection, localization and measurement of the nucleic acids encoding for the production of the proteins of the present invention, and also for the detection, localization and measurement of the proteins of the present invention. These nucleotides and antibodies can be combined with other reagents in kits for the purposes of detection, localization and measurement. These kits are useful for diagnosis and prognosis of conditions involving cellular proliferation associated with production of reactive oxygen intermediates.

The present invention solves the problems described above by providing a composition comprising the nucleotide sequence SEQ ID NO: 1 and fragments thereof and conservative substitutions thereof. The present invention also provides a composition comprising the nucleotide sequence SEQ ID NO: 3 and fragments thereof and conservative substitutions thereof. The present invention

provides a composition comprising the protein SEQ ID NO: 2, and fragments and conservative substitutions thereof, encoded by the nucleotide sequence SEQ ID NO: 1 and fragments and conservative substitutions thereof. The present invention provides a composition comprising the protein SEQ ID NO: 4 and fragments and conservative substitutions thereof, encoded by the nucleotide sequence SEQ ID NO: 3 and fragments and conservative substitutions thereof. Preferred protein fragments include, but are not limited to, SEQ ID NO: 31 and SEQ ID NO: 32.

The present invention also provides vectors containing the nucleotide sequences SEQ ID NO: 1, and SEQ ID NO: 3 or fragments thereof. The present invention also provides cells transfected with these vectors.

In addition, the present invention provides cells stably transfected with the nucleotide sequence SEQ ID NO: 1 or fragments thereof. The present invention also provides cells stably transfected with the nucleotide sequence SEQ ID NO: 3 or fragments thereof.

The present invention provides cells stably transfected with the nucleotide sequence SEQ ID NO: 1 or fragments or conservative substitutions thereof, which produce the protein SEQ ID NO: 2 or fragments or conservative substitutions thereof. In addition, the present invention provides cells stably transfected with the nucleotide sequence SEQ ID NO: 3 or fragments or conservative substitutions thereof which produce the protein SEQ ID NO: 4 or fragments or conservative substitutions thereof.

The present invention provides a method for stimulating growth by administering cells stably transfected with the nucleotide sequence SEQ ID NO: 1 or fragments or conservative substitutions thereof which produce the protein SEQ ID NO: 2 or fragments or conservative substitutions thereof. The present invention also provides a method for stimulating growth by administering cells stably transfected with the nucleotide sequence SEQ ID NO: 3 or fragments or conservative substitutions thereof, which produce the protein SEQ ID NO: 4 or fragments or conservative substitutions thereof.

Specifically, the present invention provides a method for stimulating tumor formation by administering cells stably transfected with the nucleotide sequence SEQ ID NO: 1 or fragments thereof, which produce the protein SEQ ID NO: 2 or fragments thereof. The present invention also provides a method for stimulating tumor formation by administering cells stably transfected with the nucleotide sequence SEQ ID NO: 3 or fragments thereof, which produce the protein SEQ ID NO: 4 or fragments thereof. The present invention may also be used to develop anti-sense nucleotide sequences to SEQ ID NO: 1 and SEQ ID NO: 3, or fragments thereof. These anti-sense molecules may be used to interfere with translation of nucleotide sequences, such as SEQ ID NO: 1, and SEQ ID NO: 3, or fragments thereof, that encode for proteins such as SEQ ID NO: 2, SEQ ID NO: 4, or fragments thereof. Administration of these anti-sense molecules, or vectors encoding for anti sense molecules, to humans and animals, would interfere with production of proteins such as SEQ ID NO: 2, SEQ ID NO: 4, or fragments thereof, thereby decreasing production of ROIs and inhibiting cellular proliferation. These methods are useful in producing animal models for use in study of tumor development, cuticle formation and vascular growth, and for study of the efficacy of treatments for affecting tumor growth, vascular growth and cuticle formation *in vivo*.

The present invention also provides a method for high throughput screening of drugs and chemicals which modulate the proliferative activity of the enzymes of the present invention or fragments or conservative substitutions thereof, thereby affecting cell division, metabolic activity, cuticle formation, fibrosis and other biological functions involving oxidative reactions. Combinatorial chemical libraries may be screened for chemicals which modulate the proliferative activity or oxidative activity of these enzymes. Drugs and chemicals may be evaluated based on their ability to modulate the enzymatic activity of the expressed or endogenous proteins, including those represented SEQ ID NO: 2 and SEQ ID NO: 4 or fragments or conservative substitutions thereof. Endogenous proteins may be obtained from many different tissues or cells, such as colon cells. Drugs may also be evaluated based on their ability to bind to the

expressed or endogenous proteins represented by SEQ ID NO: 2 and SEQ ID NO: 4 or fragments or conservative substitutions thereof. Enzymatic activity may be NADPH- or NADH-dependent superoxide generation catalyzed by the holoprotein. Enzymatic activity may also be NADPH- or NADH-dependent diaphorase activity catalyzed by either the holoprotein or the flavoprotein domain.

By flavoprotein domain, is meant approximately the C-terminal half of the enzymes shown in SEQ ID NO: 2 and SEQ ID NO: 4, or fragments or conservative substitutions thereof, (approximately the C-terminal 265 amino acids). This fragment of gp91phox has NADPH-dependent reductase activity towards cytochrome c, nitrobluetetrazolium and other dyes. Expressed proteins or fragments thereof can be used for robotic screens of existing combinatorial chemical libraries. While not wanting to be bound by the following statement, it is believed that the NADPH or NADH binding site and the FAD binding site are useful for evaluating the ability of drugs and other compositions to bind to the duox enzymes or fragments or conservative substitutions thereof, or to modulate their enzymatic activity. The use of the holoprotein or the C-terminal half or end regions are preferred for developing a high throughput drug screen. Additionally, the N-terminal one-third of the duox domain (the peroxidase domain) may also be used to evaluate the ability of drugs and other compositions to inhibit the peroxidase activity, and for further development of a high throughput drug screen.

The present invention also provides antibodies directed to the oxidative enzymes such as SEQ ID NO: 2 and SEQ ID NO: 4 and fragments or conservative substitutions thereof. Preferred protein fragments include, but are not limited to, SEQ ID NO: 31 and SEQ ID NO: 32. The antibodies of the present invention are useful for a variety of purposes including localization, detection and measurement of the proteins SEQ ID NO: 2 and SEQ ID NO: 4 and fragments or conservative substitutions thereof. The antibodies may be employed in kits to accomplish these purposes. These antibodies may also be linked to cytotoxic agents for selected killing of cells. The term antibody is meant to include any class of antibody such as IgG, IgM

and other classes. The term antibody also includes a completely intact antibody and also fragments thereof, including but not limited to Fab fragments and Fab + Fc fragments.

The present invention also provides the nucleotide sequences SEQ ID NO: 1 and SEQ ID NO: 3 and fragments or conservative substitutions thereof. These nucleotides are useful for a variety of purposes including localization, detection, and measurement of messenger RNA involved in synthesis of the proteins represented as SEQ ID NO: 2 and SEQ ID NO: 4 and fragments or conservative substitutions thereof. These nucleotides may also be used in the construction of labeled probes for the localization, detection, and measurement of nucleic acids such as messenger RNA or alternatively for the isolation of larger nucleotide sequences containing the nucleotide sequences shown in SEQ ID NO: 1, and SEQ ID NO: 3 or fragments or conservative substitutions thereof. These nucleotide sequences may be used to isolate homologous strands from other species using techniques known to one of ordinary skill in the art. These nucleotide sequences may also be used to make probes and complementary strands.

Most particularly, the present invention involves a method for modulation of growth by modifying the proteins represented as SEQ ID NO: 2 and SEQ ID NO: 4 or fragments or conservative substitutions thereof.

The term "mitogenic regulators" is used herein to mean any molecule that acts to affect cell division.

The term "animal" is used herein to mean humans and non-human animals of both sexes.

The terms "a", "an" and "the" as used herein are defined to mean one or more and include the plural unless the context is inappropriate.

"Proteins", "peptides," "polypeptides" and "oligopeptides" are chains of amino acids (typically L-amino acids) whose alpha carbons are linked through peptide bonds formed by a condensation reaction between the carboxyl group of the alpha carbon of one amino acid and the amino group of the alpha carbon of another amino acid. The terminal amino acid at one end of the chain (*i.e.*, the

amino terminal) has a free amino group, while the terminal amino acid at the other end of the chain (*i.e.*, the carboxy terminal) has a free carboxyl group. As such, the term "amino terminus" (abbreviated N-terminus) refers to the free alpha-amino group on the amino acid at the amino terminal of the protein, or to the alpha-amino group (imino group when participating in a peptide bond) of an amino acid at any other location within the protein. Similarly, the term "carboxy terminus" (abbreviated C-terminus) refers to the free carboxyl group on the amino acid at the carboxy terminus of a protein, or to the carboxyl group of an amino acid at any other location within the protein.

Typically, the amino acids making up a protein are numbered in order, starting at the amino terminal and increasing in the direction toward the carboxy terminal of the protein. Thus, when one amino acid is said to "follow" another, that amino acid is positioned closer to the carboxy terminal of the protein than the preceding amino acid.

The term "residue" is used herein to refer to an amino acid (D or L) or an amino acid mimetic that is incorporated into a protein by an amide bond. As such, the amino acid may be a naturally occurring amino acid or, unless otherwise limited, may encompass known analogs of natural amino acids that function in a manner similar to the naturally occurring amino acids (*i.e.*, amino acid mimetics). Moreover, an amide bond mimetic includes peptide backbone modifications well known to those skilled in the art.

Furthermore, one of skill will recognize that, as mentioned above; individual substitutions, deletions or additions which alter, add or delete a single amino acid or a small percentage of amino acids (typically less than 5%, more typically less than 1%) in an encoded sequence are conservatively modified variations where the alterations result in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art. The following six groups each contain amino acids that are conservative substitutions for one another:

1) Alanine (A), Serine (S), Threonine (T);

2) Aspartic acid (D), Glutamic acid (E);

3) Asparagine (N), Glutamine (Q);

4) Arginine (R), Lysine (K);

5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and

6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

When the peptides are relatively short in length (*i.e.*, less than about 50 amino acids), they are often synthesized using standard chemical peptide synthesis techniques. Solid phase synthesis in which the C-terminal amino acid of the sequence is attached to an insoluble support followed by sequential addition of the remaining amino acids in the sequence is a preferred method for the chemical synthesis of the antigenic epitopes described herein. Techniques for solid phase synthesis are known to those skilled in the art.

Alternatively, the antigenic epitopes described herein are synthesized using recombinant nucleic acid methodology. Generally, this involves creating a nucleic acid sequence that encodes the peptide or protein, placing the nucleic acid in an expression cassette under the control of a particular promoter, expressing the peptide or protein in a host, isolating the expressed peptide or protein and, if required, renaturing the peptide or protein. Techniques sufficient to guide one of skill through such procedures are found in the literature.

When several desired protein fragments or peptides are encoded in the nucleotide sequence incorporated into a vector, one of skill in the art will appreciate that the protein fragments or peptides may be separated by a spacer molecule such as, for example, a peptide, consisting of one or more amino acids. Generally, the spacer will have no specific biological activity other than to join the desired protein fragments or peptides together, or to preserve some minimum distance or other spatial relationship between them. However, the constituent amino acids of the spacer may be selected to influence some property of the molecule such as the folding, net charge, or hydrophobicity. Nucleotide sequences encoding for the production of residues which may be useful in purification of the expressed recombinant protein may be built into the vector. Such sequences are

known in the art. For example, a nucleotide sequence encoding for a poly histidine sequence may be added to a vector to facilitate purification of the expressed recombinant protein on a nickel column.

Once expressed, recombinant peptides, polypeptides and proteins can be purified according to standard procedures known to one of ordinary skill in the art, including ammonium sulfate precipitation, affinity columns, column chromatography, gel electrophoresis and the like. Substantially pure compositions of about 50 to 99% homogeneity are preferred, and 80 to 95% or greater homogeneity are most preferred for use as therapeutic agents.

One of skill in the art will recognize that after chemical synthesis, biological expression or purification, the desired proteins, fragments thereof and peptides may possess a conformation substantially different than the native conformations of the proteins, fragments thereof and peptides. In this case, it is often necessary to denature and reduce protein and then to cause the protein to re-fold into the preferred conformation. Methods of reducing and denaturing proteins and inducing re-folding are well known to those of skill in the art.

The genetic constructs of the present invention include coding sequences for different proteins, fragments thereof, and peptides. The genetic constructs also include epitopes or domains chosen to permit purification or detection of the expressed protein. Such epitopes or domains include DNA sequences encoding the glutathione binding domain from glutathione S-transferase, hexa-histidine, thioredoxin, hemagglutinin antigen, maltose binding protein, and others commonly known to one of skill in the art. The preferred genetic construct includes the nucleotide sequences of SEQ ID NO: 1 and SEQ ID NO: 3 or fragments or conservative substitutions thereof. It is to be understood that additional or alternative nucleotide sequences may be included in the genetic constructs in order to encode for the following: a) multiple copies of the desired proteins, fragments thereof, or peptides; b) various combinations of the desired proteins, fragments thereof, or peptides; and c) conservative modifications of the desired proteins, fragments thereof, or peptides, and combinations thereof. Still another preferred

protein of the present invention is human duox2 (SEQ ID NO: 2) protein, and fragments or conservative substitutions thereof, as encoded by SEQ ID NO: 1 and fragments or conservative substitutions thereof. Another preferred protein of the present invention is Ce Duox 1 (SEQ ID NO:4) protein and fragments or conservative substitutions thereof, as encoded by SEQ ID NO: 3 and fragments or conservative substitutions thereof.The nucleotide sequences of the present invention may also be employed to hybridize to nucleic acids such as DNA or RNA nucleotide sequences under high stringency conditions which permit detection, for example, of alternately spliced messages.

The genetic construct is expressed in an expression system such as in NIH 3T3 cells using recombinant sequences in a pcDNA-3 vector (Invitrogen, Carlsbad, CA) to produce a recombinant protein. Preferred expression systems include but are not limited to Cos-7 cells, insect cells using recombinant baculovirus, and yeast. It is to be understood that other expression systems known to one of skill in the art may be used for expression of the genetic constructs of the present invention. A preferred protein of the present invention is referred to herein as human duox2, or fragments or conservative substitutions thereof, which has the amino acid sequence set forth in SEQ ID NO:2, or an amino acid sequence having amino acid substitutions as defined in the definitions that do not significantly alter the function of the recombinant protein in an adverse manner. Another preferred protein of the present invention is Ce Duox 1 (SEQ ID NO: 4) or fragments or conservative substitutions thereof, as encoded by SEQ ID NO: 3 and fragments or conservative substitutions thereof.

Terminology

As described herein, the term "human duox2" refers to a protein comprising an amino acid sequence as set forth in SEQ ID NO:2, or a fragment or conservative substitution thereof, and encoded by the nucleotide sequence as set forth in SEQ ID NO:1, or a fragment or conservative substitution thereof. Ce duox refers to duox from *C. elegans* or a fragment or conservative substitution thereof.

Construction of the Recombinant Gene

The desired gene is ligated into a transfer vector, such as pcDNA3, and the recombinants are used to transform host cells such as Cos-7 cells. It is to be understood that different transfer vectors, host cells, and transfection methods may be employed as commonly known to one of ordinary skill in the art. Two desired genes for use in transfection are shown in SEQ ID NO: 1, and SEQ ID NO: 3. For example, lipofectamine-mediated transfection and *in vivo* homologous recombination is used to introduce the duox1 gene into NIH 3T3 cells.

The synthetic gene is cloned and the recombinant construct containing duox gene is produced and grown in confluent monolayer cultures of a Cos-7 cell line. The expressed recombinant protein is then purified, preferably using affinity chromatography techniques, and its purity and specificity determined by known methods.

A variety of expression systems may be employed for expression of the recombinant protein. Such expression methods include, but are not limited to the following: bacterial expression systems, including those utilizing *E. coli* and *Bacillus subtilis*; virus systems; yeast expression systems; cultured insect and mammalian cells; and other expression systems known to one of ordinary skill in the art.

Transfection of Cells

It is to be understood that the vectors of the present invention may be transfected into any desired cell or cell line. Both *in vivo* and *in vitro* transfection of cells are contemplated as part of the present invention. Preferred cells for transfection include but are not limited to the following: fibroblasts (possibly to enhance wound healing and skin formation), granulocytes (possible benefit to increase function in a compromised immune system as seen in AIDS, and aplastic anemia), muscle cells, neuroblasts, stem cells, bone marrow cells, osteoblasts, B lymphocytes, and T lymphocytes.

Cells may be transfected with a variety of methods known to one of ordinary skill in the art and include but are not limited to the following: electroporation, gene gun, calcium phosphate, lipofectamine, and fugene, as well as adenoviral transfection systems.

Host cells transfected with the nucleic acids represented in SEQ ID NO: 1, and SEQ ID NO: 3, or fragments or conservative substitutions thereof, are used to express the proteins SEQ ID NO: 2 and SEQ ID NO: 4, respectively, or fragments or conservative substitutions thereof.

These expressed proteins are used to raise antibodies. These antibodies may be used for a variety of applications including but not limited to immunotherapy against cancers expressing one of the duox proteins, for affecting cuticle formation, and for detection, localization and measurement of the proteins shown in SEQ ID NO: 2 and SEQ ID NO: 4, or fragments or conservative substitutions thereof.

Purification and Characterization of the Expressed Protein

The proteins of the present invention can be expressed as a fusion protein with a poly histidine component, such as a hexa histidine, and purified by binding to a metal affinity column using nickel or cobalt affinity matrices. The protein can also be expressed as a fusion protein with glutathione S-transferase and purified by affinity chromatography using a glutathione agarose matrix. The protein can also be purified by immunoaffinity chromatography by expressing it as a fusion protein, for example with hemagglutinin antigen. The expressed or naturally occurring protein can also be purified by conventional chromatographic and purification methods which include anion and cation exchange chromatography, gel exclusion chromatography, hydroxylapatite chromatography, dye binding chromatography, ammonium sulfate precipitation, precipitation in organic solvents or other techniques commonly known to one of skill in the art.

Methods of Assessing Activity of Expressed Proteins

Different methods are available for assessing the activity of the expressed proteins of the present invention, including, but not limited to, the proteins represented as SEQ ID NO: 2 and SEQ ID NO: 4, substituted analogs thereof, and fragments or conservative substitutions thereof.

1. Assays of the holoprotein and fragments thereof for superoxide generation:

A. General considerations.

These assays are useful in assessing efficacy of drugs designed to modulate the activity of the enzymes of the present invention. The holoprotein may be expressed in COS-7 cells, NIH 3T3 cells, insect cells (using baculoviral technology) or other cells using methods known to one of skill in the art. Membrane fractions or purified protein are used for the assay. The assay may require or be augmented by other cellular proteins such as p47phox, p67phox, and Rac1, as well as potentially other unidentified factors (e.g., kinases or other regulatory proteins).

B. Cytochrome c reduction.

NADPH or NADH is used as the reducing substrate, in a concentration of about 100 μM. Reduction of cytochrome c is monitored spectrophotometrically by the increase in absorbance at 550 nm, assuming an extinction coefficient of 21 $mM^{-1}cm^{-1}$. The assay is performed in the absence and presence of about 10 μg superoxide dismutase. The superoxide-dependent reduction is defined as cytochrome c reduction in the absence of superoxide dismutase minus that in the presence of superoxide dismutase (Uhlinger et al. (1991) *J. Biol. Chem.* 266, 20990-20997). Acetylated cytochrome c may also be used, since the reduction of acetylated cytochrome c is thought to be exclusively via superoxide.

C. Nitroblue tetrazolium reduction.

For nitroblue tetrazolium (NBT) reduction, the same general protocol is used, except that NBT is used in place of

cytochrome c. In general, about 1 mL of filtered 0.25 % nitrotetrazolium blue (Sigma, St. Louis, MO) is added in Hanks buffer without or with about 600 Units of superoxide dismutase (Sigma) and samples are incubated at approximately 37°C. The oxidized NBT is clear, while the reduced NBT is blue and insoluble. The insoluble product is collected by centrifugation, and the pellet is re-suspended in about 1 mL of pyridine (Sigma) and heated for about 10 minutes at 100°C to solubilize the reduced NBT. The concentration of reduced NBT is determined by measuring the absorbance at 510 nm, using an extinction coefficient of 11,000 $M^{-1}cm^{-1}$. Untreated wells are used to determine cell number.

D. Luminescence.

Superoxide generation may also be monitored with a chemiluminescence detection system utilizing lucigenin (bis-N-methylacridinium nitrate, Sigma, St. Louis, MO). The sample is mixed with about 100 μM NADPH (Sigma, St. Louis, MO) and 10 μM lucigenin (Sigma, St. Louis, MO) in a volume of about 150 μL Hanks solution. Luminescence is monitored in a 96-well plate using a LumiCounter (Packard, Downers Grove, IL) for 0.5 second per reading at approximately 1 minute intervals for a total of about 5 minutes; the highest stable value in each data set is used for comparisons. As above, superoxide dismutase is added to some samples to prove that the luminescence arises from superoxide. A buffer blank is subtracted from each reading (Ushio-Fukai et al. (1996) *J. Biol. Chem.* 271, 23317-23321).

E. Assays in intact cells.

Assays for superoxide generation may be performed using intact cells, for example, the duox-transfected NIH 3T3 cells. In principle, any of the above assays can be used to evaluate superoxide generation using intact cells, for example, the duox-transfected NIH 3T3 cells. NBT reduction is a preferred assay method.

2. Assays of truncated proteins comprised of approximately the C-terminal 265 amino acid residues

While not wanting to be bound by the following statement, the truncated protein comprised of approximately the C-terminal 265 amino acid residues is not expected to generate superoxide, and therefore, superoxide dismutase is not added in assays of the truncated protein. Basically, a similar assay is established and the superoxide-independent reduction of NBT, cytochrome c, dichlorophenolindophenol, ferricyanide, or another redox-active dye is examined.

Nucleotides and Nucleic Acid Probes

The nucleotide sequences SEQ ID NO: 1 and SEQ ID NO: 3, as well as fragments or conservative substitutions thereof, and PCR primers therefor, may be used, respectively, for localization, detection and measurement of nucleic acids related to SEQ ID NO: 1 and SEQ ID NO: 3, as well as fragments or conservative substitutions thereof. SEQ ID NO 1 is also known as a nucleotide sequence encoding human duox2 in this application. SEQ ID NO: 3 is also known as a nucleotide sequence encoding Ce duox 1 in this application.

The nucleotide sequences SEQ ID NO: 1, SEQ ID NO: 3, as well as fragments or conservative substitutions thereof, may be used to create probes to isolate larger nucleotide sequences containing the nucleotide sequences SEQ ID NO: 1, SEQ ID NO: 3, respectively. The nucleotide sequences SEQ ID NO: 1, SEQ ID NO: 3, as well as fragments or conservative substitutions thereof, may also be used to create probes to identify and isolate duox proteins in other species.

The nucleic acids described herein include messenger RNA coding for production of SEQ ID NO: 2, SEQ ID NO: 4, and fragments thereof. Such nucleic acids include but are not limited to cDNA probes. These probes may be labeled in a variety of ways known to one of ordinary skill in the art. Such methods include but are not limited to isotopic and non-isotopic labeling. These probes may be used for *in situ* hybridization for localization of nucleic acids such as mRNA encoding for SEQ ID NO: 2, and SEQ ID NO: 4, and fragments or conservative substitutions thereof. Localization may be

performed using *in situ* hybridization at both ultrastructural and light microscopic levels of resolution using techniques known to one of ordinary skill in the art.

These probes may also be employed to detect and quantitate nucleic acids and mRNA levels using techniques known to one of ordinary skill in the art including but not limited to solution hybridization.

Antibody Production

The proteins shown in SEQ ID NO: 2, SEQ ID NO: 4 SEQ ID NO: 31 and SEQ ID NO: 32, or fragments or conservative substitutions thereof, are combined with a pharmaceutically acceptable carrier or vehicle to produce a pharmaceutical composition and administered to animals for the production of polyclonal antibodies using methods known to one of ordinary skill in the art. The preferred animals for antibody production are rabbits and mice. Other animals may be employed for immunization with these proteins or fragments thereof. Such animals include, but are not limited to the following; sheep, horses, pigs, donkeys, cows, monkeys and rodents such as guinea pigs and rats.

The terms "pharmaceutically acceptable carrier or pharmaceutically acceptable vehicle" are used herein to mean any liquid including but not limited to water or saline, oil, gel, salve, solvent, diluent, fluid ointment base, liposome, micelle, giant micelle, and the like, which is suitable for use in contact with living animal or human tissue without causing adverse physiological responses, and which does not interact with the other components of the composition in a deleterious manner.

The pharmaceutical compositions may conveniently be presented in unit dosage form and may be prepared by conventional pharmaceutical techniques. Such techniques include the step of bringing into association the active ingredient and the pharmaceutical carrier(s) or excipient(s). In general, the formulations are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers.

Formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example, sealed ampules and vials, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example, water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets commonly used by one of ordinary skill in the art.

Preferred unit dosage formulations are those containing a dose or unit, or an appropriate fraction thereof, of the administered ingredient. It should be understood that in addition to the ingredients, particularly mentioned above, the formulations of the present invention may include other agents commonly used by one of ordinary skill in the art.

The pharmaceutical composition may be administered through different routes, such as oral, including buccal and sublingual, rectal, parenteral, aerosol, nasal, intramuscular, subcutaneous, intradermal, and topical. The pharmaceutical composition of the present invention may be administered in different forms, including but not limited to solutions, emulsions and suspensions, microspheres, particles, microparticles, nanoparticles, and liposomes. It is expected that from about 1 to 7 dosages may be required per immunization regimen. Initial injections may range from about 0.1 μg to 1 mg, with a preferred range of about 1 μg to 800 μg, and a more preferred range of from approximately 25 μg to 500 μg. Booster injections may range from 0.1 μg to 1 mg, with a preferred range of approximately 1 μg to 800 μg, and a more preferred range of about 10 μg to 500 μg.

The volume of administration will vary depending on the route of administration and the size of the recipient. For example, intramuscular injections may range from about 0.1 ml to 1.0 ml.

The pharmaceutical composition may be stored at temperatures of from about 4°C to -100°C. The pharmaceutical composition may also be stored in a lyophilized state at different temperatures including room temperature. The pharmaceutical composition may be sterilized through conventional means known to one of ordinary skill in the art. Such means include, but are not limited to filtration, radiation and heat. The pharmaceutical composition of the present invention may also be combined with bacteriostatic agents, such as thimerosal, to inhibit bacterial growth.

Adjuvants

A variety of adjuvants known to one of ordinary skill in the art may be administered in conjunction with the protein in the pharmaceutical composition. Such adjuvants include, but are not limited to the following: polymers, co-polymers such as polyoxyethylene-polyoxypropylene copolymers, including block co-polymers; polymer P1005; Freund's complete adjuvant (for animals); Freund's incomplete adjuvant; sorbitan monooleate; squalene; CRL-8300 adjuvant; alum; QS 21, muramyl dipeptide; trehalose; bacterial extracts, including mycobacterial extracts; detoxified endotoxins; membrane lipids; or combinations thereof.

Monoclonal antibodies can be produced using hybridoma technology in accordance with methods well known to those skilled in the art. The antibodies are useful as research or diagnostic reagents or can be used for passive immunization. The composition may optionally contain an adjuvant.

The polyclonal and monoclonal antibodies useful as research or diagnostic reagents may be employed for detection and measurement of SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 31 and SEQ ID NO: 32, and fragments or conservative substitutions thereof. Such antibodies may be used to detect these proteins in a biological sample, including but not limited to samples such as cells, cellular extracts, tissues, tissue extracts, biopsies, tumors, and biological fluids. Such detection capability is useful for detection of disease related to these proteins to facilitate diagnosis and prognosis and to suggest possible treatment alternatives.

Detection may be achieved through the use of immunocytochemistry, ELISA, radioimmunoassay or other assays as commonly known to one of ordinary skill in the art. The duox proteins, including the hduox2 and Ce-duox proteins of the present invention, or fragments or conservative substitutions thereof, may be labeled through commonly known approaches, including but not limited to the following: radiolabeling, dyes, magnetic particles, biotin-avidin, fluorescent molecules, chemiluminescent molecules and systems, ferritin, colloidal gold, and other methods known to one of skill in the art of labeling proteins.

Administration of Antibodies

The antibodies directed to the proteins shown as SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 31, SEQ ID NO: 32 , or directed to fragments or conservative substitutions thereof, may also be administered directly to humans and animals in a passive immunization paradigm. Antibodies directed to extracellular portions of SEQ ID NO: 2, and SEQ ID NO: 4, bind to these extracellular epitopes. Attachment of labels to these antibodies facilitates localization and visualization of sites of binding. Attachment of molecules such as ricin or other cytotoxins to these antibodies helps to selectively damage or kill cells expressing SEQ ID NO: 2, and SEQ ID NO: 4, or fragments thereof.

Kits

The present invention includes kits useful with the antibodies, nucleic acids, nucleic acid probes, labeled antibodies, labeled proteins or fragments thereof for detection, localization and measurement of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, or combinations and fragments or conservative substitutions thereof.

Kits may be used for immunocytochemistry, *in situ* hybridization, solution hybridization, radioimmunoassay, ELISA, Western blots, quantitative PCR, and other assays for the detection, localization and measurement of these nucleic acids, proteins or fragments thereof using techniques known to one of skill in the art.

The nucleotide sequences shown in SEQ ID NO: 1, and SEQ ID NO: 3, or fragments thereof, may also be used under high stringency conditions to detect alternately spliced messages related to SEQ ID NO: 1, and SEQ ID NO: 3, or fragments thereof, respectively.

The diagnostic kits may measure or detect the relative expression of the duox proteins described herein (i.e. human duox1 and/or human duox2 and ce-duox).

Fragments of SEQ ID NO: 1, and SEQ ID NO: 3, containing the relevant hybridizing sequence can be synthesized onto the surface of a chip array. RNA samples, e.g., from tumors, are then fluorescently tagged and hybridized onto the chip for detection. This approach may be used diagnostically to characterize tumor types and to tailor treatments and/or provide prognostic information. Such prognostic information may have predictive value concerning disease progression and life span, and may also affect choice of therapy.

The present invention is further illustrated by the following examples, which are not to be construed in any way as imposing limitations upon the scope thereof. On the contrary, it is to be clearly understood that resort may be had to various other embodiments, modifications, and equivalents thereof, which, after reading the description herein, may suggest themselves to those skilled in the art without departing from the spirit of the present invention.

EXAMPLE 1

Cloning of cDNA for human Duox 2

A 535-base portion of an expressed sequence tag (EST zc92h03.rl; Genbank accession no. W52750) from human pancreatic islet was identified using the amino-acid sequence of human gp91*phox* as a query in a Blast search. The bacterial strain #595758 containing the EST sequence zc92h03.rl in the pBluescript SK-vector was purchased from ATCC (Rockville, MD). The DNA was sequenced using primers to T7 and T3 vector promoters as well as sequence-specific internal primers. The EST encoded a 440 amino

acid partial cDNA exhibiting 24.4 % identity to gp91*phox*, including a stop codon corresponding to the C-terminus of gp91*phox*. 5'- and 3'-RACE were carried out using human adult pancreas mRNA (Clontech, Palo Alto, CA) with the 5' RACE kit for Rapid Amplification of cDNA Ends version 2.0 (Gibco BRL, Gaithersburg, MD). PCR was done with specific primers: 5'-RACE: Primer 1, 5'-GAAGTGGTGGGAGGCGAAGACATA-3' (SEQ ID NO:5); Primer 2, 5'-CCTGTCATACCTGGGACGGTCTGG-3' (SEQ ID NO:6); Primer 3, 5'-GAGCACAGTGAGATGCCTGTTCAG-3' (SEQ ID NO:7); Primer 4, 5'-GGAAGGCAGCAGAGAGCAATGATG-3' (SEQ ID NO:8); Primer 5, 5'-AGGTGGGATGCGGATGTTGAG-3' (SEQ ID NO:9) (for nested PCR); 3'-RACE Primer 6, 5'-ACATCTGCGAGCGGCACTTCCAGA-3' (SEQ ID NO:10); Primer 7, 5'-AGCTCGTCAACAGGCAGGACCGAGC-3' (SEQ ID NO:11); Primer 8, 5'-TCTCCATCAGAATCCACCTTAGGC-3' (SEQ ID NO:12) (for nested PCR). To complete the sequence, 5'-RACE was carried out using human thyroid Marathon-ready cDNA (Clontech, Palo Alto, CA) with primer 3 and adapter primer AP1, and primer 5 and adapter primer AP2. These procedures resulted in an additional 3.7 kb 5' region and a 1.5 kb 3' region.

The cDNA for h-Duox2 showed a 4647 base pair open reading frame (Genebank #AF267981) that is predicted to encode a protein of 1548 amino acids (175 kDa), and contained a consensus Kozak sequence, GGCATGC (SEQ ID NO: 13), at the translation start codon. The Duox2 cDNA sequence is a larger form of a gp91*phox* homolog previously identified as an NADPH-oxidase in thyroid and termed $p138^{Tox}$; the latter sequence did not contain the a peroxidase homology domain (Dupuy *et al.*, 1999). h-Duox1 and h-Duox2 were 77% identical at the amino acid level.

EXAMPLE 2

Identification of genes for Ce-Duox1 and Ce-Duox2, Cloning of the cDNA for Ce-Duox1

A BLAST search using the cDNA sequence of human gp91*phox* identified two putative homologues (Genbank #s AF043697 and AF003130) in the genomic sequence of *C. elegans*, both near the

end of chromosome I and separated by ~6 Kb. Based on the gene sequence, PCR primers were designed to amplify two overlapping portions of the Ce-Duox1 gene, one extending from the 5' end and one extending from the 3' end. Primers were 5'-ATTCGTCGACAAATGCGCTCAAAACATGTGCTGT-3' (SEQ ID NO: 14) and 5'-AACTTTGTGGATCAAAGTTAGCG-3' (SEQ ID NO: 15) for the 5' region, and 5'-TTGGATTAGCATTTTGCTATGGAA-3' and (SEQ ID NO: 16) 5'-GAGCGGCCGCGAACGTTTCAAAGCGATGTGCA-3' (SEQ ID NO: 17) for the 3' region. PCR was carried out using a random primed *C. elegans* cDNA library in lACT (obtained from R. Barstead, Oklahoma Medical Research Foundation) under the following conditions: denaturation at 95°C for 30 seconds; annealing at 59°C for 30 seconds; extension at 72°C for 1 minute. The 5' piece and the 3' piece were digested with Dra III and ligated to produce the full length Ce-Duox1 cDNA. The full length Ce-Duox1 cDNA was inserted into the pBluescript SK-vector and was sequenced using T7 and T3 vector primers and sequence specific primers.

Duox homologs in C. elegans. A BLAST search of the *C. elegans* genomic database using as a query the protein sequence of gp91*phox* identified two homologous genes contained in cosmids F56C11 and F53G12. The Ce-Duox1 conceptual transcript (Genebank # AF043697) is predicted to be 8197 bp before splicing, to contain 19 exons, and to encode a protein of 1506 amino acids. Cloning of the cDNA for Ce-Duox1 (Genebank #AF229855) revealed a cDNA of 4491 bp (1497 amino acids), which differed somewhat from the conceptual cDNA obtained from the gene structure due to inaccuracies in the predicted intron-exon junctions. The second transcript, Ce-Duox2 (Genebank #AF043697), is predicted to be 5308 bp before splicing, to contain 16 exons, and to encode a 1313 amino acid protein. Alignment by homology of the genomic sequences of Ce-Duox1 and Ce-Duox2 identified two new exons 5' of the first predicted exon of Duox2 that were highly homologous to the second and third exons of Duox1, but an exon of Duox2 homologous to exon1 of Duox1 could not be identified by homology. The predicted

amino acid sequences of both Ce-Duox1 and Ce-Duox2 show approximately 30% identity with h-Duox1 and h-Duox2 (Fig. 1 and 2A). Ce-Duox1 also contains the same domains as h-Duox1/2 (see below) and is roughly the same size. However, Ce-Duox2 contains a stop codon which should eliminate the extreme C-terminal portion of the protein, which includes a segment of the pyridine nucleotide binding site. Thus, while Ce-Duox2 should contain intact peroxidase and calmodulin-like domains, it is not predicted to encode a functioning NADPH-oxidase domain (see Fig. 1). Except for this C-terminal region, Ce-Duox2 is 94% identical to Ce-Duox1 at the amino acid level. Both Ce-Duox1 and Ce-Duox2 are located near the end of chromosome I, separated by only 6 kb and in opposite orientations. The high degree of sequence identity and retention of intron structure (data not shown), as well as the location of both near the end of a chromosome are consistent with a recent gene duplication.

EXAMPLE 3

Analysis of primary structure; Domain Organization and Sequence Comparisons Among gp91phox, h-Duox2, Ce-Duox1 and Ce-Duox2.

Export signal sequences were predicted according to Nielsen *et al.*, 1997. Transmembrane alpha helices were predicted according to Sonnhammer *et al.*, 1998. Both methods are available on the internet at the Center for Biological Sequence Analysis (http://www.cbs.dtu.dk/services/). Multiple sequence alignments phylogenetic analysis were carried out using the clustal method, using Megalign software (DNASTAR).

The domain structure and transmembrane regions in gp91*phox*, h-Duox1, hDuox2, Ce-Duox1 and Ce-Duox2 are diagrammed in Fig. 1. Duox enzymes are homologous to gp91*phox* in their C-termini (see http://www.biochem.emory.edu/Lambeth/gp91_homology.pdf for an alignment of these regions). Nox1 (Suh *et al.*, 1999), which is the same size as gp91*phox*, is more closely related to gp91*phox* (54 % identical) than is the NADPH-oxidase domain of hDuox1 or hDuox2 (~26 % identical to gp91*phox*). However, h-Duox1 and hDuox2 are more closely related to Ce-Duox1 within the NADPH-oxidase domain

(~39 % identical). Within the putative FAD binding regions and NADPH binding regions, homologs share considerably higher homology, ranging from 60% to 90%, depending on the region. This includes the canonical dinucleotide binding helix GXGXXP (SEQ ID NO: 18). In gp91*phox*, Nox1, h-Duox1, and h-Duox2 this sequence is followed by F, which is present in many NADPH-specific flavoproteins, while in the *C. elegans* proteins, F is conservatively replaced with Y.

Duox proteins have additional regions that are not present in gp91*phox*. A central region contains two EF hand calcium binding sequences, as indicated in Fig. 1. The canonical residues involved in calcium ligation are well conserved in h-Duox1 and h-Duox2, but are poorly conserved in Ce-Duox1 and Ce-Duox2, suggesting that the function of this region may have evolved away from calcium binding in nematodes.

Surprisingly, the N-terminal third of Duox proteins is homologous to peroxidases including myeloperoxidase, eosinophil peroxidase, thyroid peroxidase, lactoperoxidase and sea urchin ovoperoxidases (Fig. 2A and 2B). Overall, the identity with peroxidases within the entire region is approximately 20%, but sub-regions show considerably higher homology. The Duox enzymes represent a distinct group within the peroxidase family (Fig. 2B), and phylogenetically, this group is marginally more closely related to sea urchin ovoperoxidases. Within the peroxidase homology region, only 2 of the 12 cysteine residues involved in the six intra-chain disulfide bonds, which are conserved in the four homologous mammalian peroxidases, are present in Duox proteins (not shown). In addition, the asparagine-linked glycosylation sites found in MPO are not present in Ce-Duox1 or Ce-Duox2. A calcium binding site in MPO (aspartate 263 and residues 335 to 341, superior double bar in Figure 2A) (Zeng and Fenna, 1992) is well conserved in the Duox family proteins, including 3 of the 4 candidate calcium liganding residues (filled triangles).

The extreme N-terminal 21 amino acids of Ce-Duox1 contains a secretory signal peptide sequence (Fig. 1), implying that the N-terminal peroxidase domain is in a compartment that is

transmembrane to the cytosol (e.g., extracellular or within a secretory vesicle). In addition, hydropathy plots reveal that the proteins contain a highly hydrophobic region corresponding to the N-terminal third of gp91*phox*. This region can be modelled as a cluster of 6 transmembrane alpha helices, as indicated in Fig. 1. An additional transmembrane helical region is present between the peroxidase homology domain and the calmodulin-like domain.

EXAMPLE 4

PCR Detection of mRNA for Human Duox, Tissue Distribution of h-Duox mRNA.

Based on the cloned h-Duox1 and hDuox2 cDNA sequence, we designed specific primers (Duox1: 5'-GCAGGACATCAACCCTGCACTCTC-3' (SEQ ID NO:19); 5'-CTGCCATCTACCACACGGATCTGC-3' (SEQ ID NO:20); Duox2: 5'-GCCCTCAACCTAAGCAGCTCACAACTG-3' (SEQ ID NO:21) ; 5'-GAGCACAGTGAGATGCCTGTTCAG-3') (SEQ ID NO:22) which were used to determine the tissue expression patterns of Duox1 and Duox2 using Human Multiple Tissue PCR Panels and human thyroid gland Marathon-Ready cDNA (Clontech, Palo Alto, CA). PCR conditions were: 95°C for 30 s, 65°C for 20 s, 72°C for 30 s, 35 cycles.

h-Duox1 mRNA was distributed among a variety of adult tissues, with highest expression in lung and thyroid, but with significant expression also seen in placenta, testis, and prostate with detectable expression in pancreas and heart. h-Duox1 mRNA was also widely expressed in fetal tissues, where it was abundant in lung. In addition, we observed significant expression in a variety of fetal tissues and in adult colon, with detectable expression in kidney, liver, lung, pancreas, prostate and testis.

h-Duox2 mRNA was distributed among a variety of adult tissues, with highest expression in colon, testis, pancreas and thyroid. h-Duox2mRNA was also widely expressed in fetal tissues, where it was abundant in lung, liver, kidney, and heart, and thyroid. We also observed significant expression in fetal skeletal muscle and thymus.

EXAMPLE 5

RNA interference (RNAi) in C. elegans Phenotypes of C. elegans RNAi Ce-Duox animals

To gain insights regarding the biological function of Duox enzymes, we used the reverse genetic tool, RNA interference (RNAi), to "knock out" Duox in *C. elegans* (Fire *et al.*, 1998). This technique involves injection of double stranded RNA (dsRNA) encoding a segment of Ce-Duox1 or Ce-Duox2 into gonads of *C. elegans* wild type hermaphrodites. Injected animals were then allowed to lay eggs, the harvested eggs were allowed to develop, and the progeny were observed for phenotypes. This procedure specifically diminishes or eliminates the expression of the gene of interest.

RNA was transcribed from either pBluescript.Duox2, pBluescript.E17Duox1 or pBluescript.E18+19Duox1. For pBluescript.Duox2, Exon 10 of Ce-Duox2 was amplified by PCR from genomic DNA using the forward primer 5'-GCTAGAGCTCTTCAGTTTGCTATGGAATTGGC-3' (SEQ ID NO:23) and reverse primer 5'-CATAAAGGATGAGGAGAATTCTGTG-3' (SEQ ID NO:24). The 457-bp fragment generated was digested with SstI and EcoRI and subcloned into pBluescript. For pBluescript.E17Duox1, Exon 17 of Duox1 was amplified by PCR from genomic DNA using the forward primer 5'-GCTAGAGCTCGGCTACTACTACGTTGTTGGACC-3' (SEQ ID NO:25) and the reverse primer 5'-GACTGAAGGACTTGTGGAACGTCTGAGTGAC-3' (SEQ ID NO:26) . The 659 bp fragment generated was digested with SstI and EcoRI and sub cloned into pBluescript. For pBluescript.E18+19Duox1, Exons 18 and 19 of Ce-Duox1 were amplified by PCR from a randomly primed *C. elegans* cDNA library (obtained from R. Barstead, Oklahoma Medical Research Foundation) using the forward primer 5'-GCTAGAGCTCACATTTGCGAGAAGCACTTCCG-3' (SEQ ID NO: 27) and the reverse primer 5'-GTGTGAATTCAGCGATGTGCAAATGAAGGAGC-3' (SEQ ID NO:- 28). The 266 bp fragment generated was digested with SstI and EcoRI and subcloned into pBluescript. Plasmids were linearized with

either Sst1 or EcoR1 and transcription was carried out using T3 and T7 RNA polymerase (Promega) in separate reactions. Sense and antisense single-stranded RNAs were combined in equal concentrations, and incubated for 10 min at 68°C followed by a 30 min incubation at 37°C to form double stranded RNA (dsRNA). dsRNAs were injected into the gonads of N2 hermaphrodite *C. elegans* as described in detail in the following paragraph Injected animals were allowed to recover and lay eggs for ~20 h after injection, transferred to individual plates, and allowed to lay eggs for a second 24 h period. The F1 progeny resulting from this second period of egg laying were evaluated. Phenotypes were observed in >90% of F1 animals.

Phenotypes of C. elegans RNAi Ce-Duox animals

dsRNA corresponding to three distinct regions of Ce-Duox1 and Duox2 were used in separate experiments. The first two correspond to regions of identity between Ce-Duox1 and Ce-Duox2 and are predicted to block the expression of both forms of Duox. The third dsRNA corresponds to the extreme C-terminus of Ce-Duox1, which does not have a counterpart in Ce-Duox2, and therefore blocks only the expression of Ce-Duox1. All three dsRNA forms resulted in the same range of phenotypes. In replicate experiments, the percentage of animals exhibiting any given phenotype was somewhat variable, probably due to differences in amount of RNAi or site of injection. However, in a typical experiment, greater than 90% of the animals were affected by one or more phenotypes. In a typical experiment, phenotypes included the presence of large superficial blisters (~50% of animals) and short or "dumpy" animals (~35% of animals), and animals with retained eggs or larvae (not shown). In addition, while wild type animals showed a dark appearance, more than 80% of RNAi animals were translucent. Around half of RNAi animals showed an inability to move on plates in a normal serpentine manner: affected animals were either completely paralyzed or moved only the anterior region, clearing a localized swath of *E. coli* in the vicinity of the head.

Similar phenotypes in *C. elegans* have been described previously and are associated with mutations in the collagen biosynthetic pathway (Levy *et al.*, 1993; Kramer, 1997; Johnstone, 2000). Several genes that encode cuticle collagens, when mutated, result in Bli ("blister"), Dpy ("dumpy", short fat worm), Rol ("roller", helical motion instead of a flat, sinusoidal motion), or Sqt ("squat", generally rollers as larvae and dumpy as adults) phenotypes. The genetics of this process are complex, since for some genes, different mutations in the same gene give rise to different phenotypes, and sometimes the phenotypes are combined (e.g. "dumpy roller"). In nematodes, collagen along with several other proteins provide the major components of cuticle, an extracellular matrix which acts as an exoskeleton.

In a global analysis of expression of all *C. elegans* genes using oligonucleotide arrays (Hill *et al.*, 2000), Ce-Duox1 was expressed at low levels (consistent with its exclusive expression in hypodermal cells) in a stage-specific manner. Expression occurred in a cyclic pattern peaking during the embryonic stage and at 36 hours, corresponding to the peak expression of other genes (Johnstone, I.L., 2000) related to collagen/cuticle biosynthesis (*col-14*, *dpy-2,-7,-10*, and *sqt-3*). A second set of collagen/cuticle-related genes (*bli-1,-2*, *col-2,-6,-17,-35,-36,-37,-41*, *dyp-13*, *sqt-1*, and *rol-6,-8*) also show peak expression at 36 hours. No significant expression of Ce-Duox2 was seen at any stage. Thus, these data are consistent with a function of Ce-Duox1 in cuticle biogenesis.

EXAMPLE 6

Generation of transgenic nematodes to study Ce-Duox1 expression

DNA from pPD96.62PRODuox1B was mixed with myo-3-GFP DNA (kindly provided by A. Fire, Carnegie Institute of Embryology, Baltimore, MD) and injected into wild type or *rol-6(su1006)* young adult hermaphrodites (Mello and Fire, 1995). Transformants were identified by screening the F1 progeny under a fluorescence dissecting microscope for green body wall muscle. These green glowing animals were stained for β-galactosidase

expression, as described below. Although pPD96.62 was expected to have driven both n –galactosidase and green fluorescent protein (GFP) expression, no fluorescence was observed outside of body wall muscle (the site of expression of the marker Myo-3-GFP). pPD96.62PRODuox1B was prepared as follows: a 3389 bp fragment was amplified by long range PCR from *C. elegans* genomic DNA using the forward primer 5'-AGTCGAAGCTTAGCATGTCAAAGTCCGGAGTTCAGT-3' (SEQ ID NO:29) and the reverse primer 5'-CTAGTGGATCCGCATTGCTCGTGCGCCTTAGAGTTT-3' (SEQ ID NO:30). The fragment included the start methionine of Ce-Duox1 and 5' untranslated sequence. The fragment was digested with HindIII and BamHI and then subcloned into pPD96.62. This construct results in the Ce-Duox1 promoter region (3389 bp) and the start methionine being inserted 5' of *E.coli* lacZ gene fused to the green fluorescence protein (GFP) reported gene.

β-Galactosidase Staining

Staining was used to detect expression of the gfp:lacZ fusion protein in transgenic worms carrying pPD96.62PRODuox1B. Reagent preparation and fixation were performed as described by Fire (1993). Vectors also incorporated a nuclear localization peptide at the N-terminus of β-galactosidase. This allows predominant staining in the nuclei of expressing cells and facilitates their identification. Nematodes were placed into individual wells of an eight well microscope slide with ~15 µl of distilled water and dried under vacuum for 2-3 min. Acetone was continuously dripped onto the dried animals for 2 min. The slide was placed in an uncovered humidity chamber, but kept dry. 10 µl of β-galactosidase stain (Fire, 1993) was layered onto each well as soon as the acetone had completely evaporated and the lid to the humidity chamber was replaced. The nematodes were then incubated at room temperature for several hours, washed several items in phosphate buffered saline, and then observed with a compound microscope.

Cellular expression of Ce-Duox1

The cellular location of Ce-Duox1 in *C. elegans* was determined by double staining with antibodies to Ce-Duox1 and to myosin A (a marker for body wall muscle cells). Ce-Duox1 was seen in larval animals in the hypodermal layer of cells immediately overlying the myosin A-containing muscle cells, and was only faintly detectable in hypodermal cells that did not overlie muscle quadrants. In adult animals, Ce-Duox1 was poorly detected (not shown). The strong signal seen in larval animals was eliminated using anti-Ce-Duox1 antibody that had been preincubated with Ce-Duox1 peptide.

EXAMPLE 7

Antibody Production and Purification

A 16 amino acid peptide corresponding to residues 340 - 355 of Ce-Duox1 was synthesized by the Emory Microchemical Facility and coupled using gluteraldehyde to keyhole limpet hemocyanin (KLH). Rabbit antibody was prepared against KLH-conjugated peptide by Lampire Biological Laboratories (Pipersville, PA) using standard protocols. Peptide (30 mg) was coupled to 1 ml of Affi-Gel 10 (Bio-Rad) for antibody immunopurification; 2 ml of serum was dialyzed against PBS and was loaded onto the Affi-Gel column preequilibrated with PBS. The column was washed with 10 ml PBS containing 1M NaCl. 0.5 ml factions of antibody were eluted with 0.1M glycine-HCl (pH 2.4) and were immediately neutralized with TRIS, pH 9. Fractions containing the highest concentration of protein were used in immunofluorescence experiments.

EXAMPLE 8

Western Blot

Nematodes were washed with M9 buffer, suspended in 0.5 ml sonication buffer (10 mM Tris HCl, pH 7.4, 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride), and sonicated 4 x 20 s. Protein was determined with the Bradford assay using bovine serum albumin as a standard. 10 µg of whole animal extract was loaded onto a 10% SDS-page gel which was then transferred to Immobilon-P membrane (Millipore). The blot was blocked for 1 hour in a solution of 5%

nonfat powdered milk and 0.05% Tween in PBS. The antibody to Ce-Duox1 was added in a 1 to 2000 dilution, incubated overnight, and the membrane was washed 3 times for 15 min with blocking solution. The blot was then developed using the SuperSignal Chemiluminescent Kit from Pierce (Rockford, Il.). A western blot of *C. elegans* protein extract showed a single band with a molecular weight of ~180,000 (data not shown).

EXAMPLE 9

Indirect Immunofluorescence.

Immunofluorescence staining of *C. elegans* was carried out as in Benian *et al.*, 1996. Mouse antibody to Myosin A was a gift from D. Miller (Miller *et al.*, 1983). Goat anti-rabbit rhodamine-conjugated antibody and goat anti-mouse FITC-conjugated antibody were used as secondary antibodies for the detection of Ce-Duox1 and Myosin A respectively. To determine non-specific binding of the Ce-Duox1 antibody, a 10 fold molar excess of Ce-Duox1 peptide was added to neutralize the antibody. Microscopy was carried out using Zeiss 510 laser scanning confocal microscope.

EXAMPLE 10

Preparation of dityrosine standard

Dityrosine standard was synthesized and purified as in Abdelrahim *et al.*, 1997 with minor modifications. Reaction products were dissolved in acidified methanol, were filtered, and directly applied to the CP-11 cellulose phosphate, eliminating the rotary evaporation step. Samples with absorption properties characteristic of dityrosine were pooled and freeze dried. For mass spectrometry, the 1 ml of dityrosine standard (0.77 mg/ml) was added to 1 ml of methanol:water (1:1) in 0.1% acetic acid.

Analysis of dityrosine and trityrosine

Nematodes were washed with M9 buffer, suspended in 0.5 ml sonication buffer (10 mM Tris HCl, pH 7.4, 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride), and sonicated 4 x 20 s. Protein was determined with the Bradford assay using bovine serum albumin as a

standard. Whole worm extracts were lyophilized and resuspended in 6 N HCl. Samples were hydrolyzed for 24 h at 110°C under vacuum, dried under vacuum and resuspended in the mobile phase for analysis by high performance liquid chromatography (HPLC) on a C18 column (0.46 x 26 cm, Fisher) using a Dionex AGP-1 HPLC instrument. The mobile phase consisted of 0.1 M KH_2PO_4 adjusted to pH 3.8 with 0.1 M phosphoric acid at a flow rate of 1 ml/min. The column eluent was monitored by fluorescence with an excitation 305-395 nm bandpass filter and an emission filter at 450 nm with a bandpass of 40 nm. To verify the identity of dityrosine, authentic dityrosine standard was added to some samples and an increase in the intensity of the putative dityrosine band was observed (data not shown).

Spectroscopic properties of di- and trityrosine.

HPLC purified samples of dityrosine and trityrosine from both *C. elegans* extracts and peroxidase domain cross-linking reactions were lyophilized and resolubilized in either 0.1 M HCl (3 ml) or 0.1 M NaOH (3 ml). Fluorescence excitation and emission spectra were obtained with a Perkin-Elmer LS-5B Luminescence Spectrometer.

Mass spectrometry

Mass spectrometry was preformed on a PE sciex API 3000 triple quadrupole mass spectrometer equipped with a turboionspray source. Dried dityrosine standard (20 mg) was reconstituted in 200 µl of H_2O. A 50 µl aliquot of this was diluted to a final volume of 1 ml with 950 µl of 5 mM ammonium acetate in MeOH and 1% acetic acid. This solution infused at a flow rate of 5 µl min^{-1}. The ionspray needle was held at +550V and –4500V for positive and negative ion analysis, respectively. These experiments identified the singly protonated (positive ion mode) and deprotonated (negative ion mode) species of the standard to be *m/z* 361.3 and 359.3 respectively as predicted.

Standard and total protein acid hydrolysate from *C. elegans* were analyzed by reverse phase LC-MS/MS. A 50 µl volume of sample was injected onto a 15 cm x 2.1 mm Supelco Discovery C18 column at a flow rate of 300 µl min^{-1}. Solvent A was 99:1 H_2O/acetic

acid and solvent B was 99:1 MeOH/acetic acid both containing 5mM ammonium acetate. The column was directly infused into the ion source of the mass spectrometer operating in positive ion mode. The column was pre-equilibrated with 100% A for 6 min followed by sample injection. The column was then washed with 100% A for 4 min and eluted with a 1 min linear gradient to 100% B, followed by a 4 min wash with 100% B. For these experiments both the precursor ions (as above for dityrosine; *m/z* 540.4/538.4 for trityrosine) and structurally distinctive breakdown ions were monitored. The transitions monitored for dityrosine were the neutral loss of both carboxyl groups, the neutral loss of both carboxyl groups and one amino group, and the neutral loss of both carboxyl groups and both amino groups (*m/z* 269.4, 252.2, and 235.0 respectively). For trityrosine, the transitions monitored were the neutral loss of a carboxyl groups, the neutral loss of a carboxyl group and one amino group, the neutral loss of two C-termini, and the neutral loss of two carboxyl groups and two amino groups (*m/z* 494.3, 477.2, 448.2, and 431.2 respectively).

Absence of tyrosine cross-linking in RNAi nematodes

Cross-linking of collagen and other cuticle proteins in nematodes occurs through di- and tri-tyrosine linkages which bridge and stabilize the proteinaceous structure (Fetterer *et al.*, 1993; Fetterer and Rhoads, 1990). Because peroxidases such as sea urchin ovoperoxidase and human myeloperoxidase carry out this reaction (Malanik and Ledvina, 1979; LaBella *et al.*, 1968; Deits *et al.*, 1984), we hypothesized that the function of Ce-Duox1 is to generate tyrosine cross-links, and that the defective cuticle in the Ce-Duox RNAi animals is due to an inability to form tyrosine cross-links. A role for an unknown peroxidase in tyrosine cross-linking in *Ascaris* was previously suggested based on studies in which tyrosine cross-linking activity was inhibited using the peroxidase inhibitors 4-amino-2,3,4 aminotriazole, phenylhydrazine, and N-acetyl tyrosine (Fetterer *et al.*, 1993). We therefore examined the wild-type and Ce-Duox1 RNAi knockout animals for di- and tri-tyrosine linkages. An HPLC profile of an acid hydrolysate of the wild-type *C. elegans* revealed a first

large peak which was identified as dityrosine based on comparison with authentic standard and mass spectral analysis, and the second peak is identified as trityrosine based on its migration on HPLC relative to dityrosine and mass spectral analysis. Based on peak areas and assuming equivalent ionization, dityrosine and tyrosine were present in a ratio of 1:200 in adult wild-type animals. In addition, the fluorescence excitation/emission maxima were determined at alkaline and acidic pH and were in good agreement with previously reported values (Jacob, *et al.* 1996). Neither the dityrosine nor the trityrosine peaks were detected in hydrolysates of Ce-Duox RNAi nematodes.

EXAMPLE 11

Participation of duox in Cuticle Biogenesis; Ultrastructural Analysis

The similarity in phenotypes among animals defective in collagen and cuticle biosynthesis compared with the RNAi Duox animals suggested that Duox participates in cuticle biogenesis. To confirm this hypothesis, electron microscopy was carried out on wild-type and RNAi animals.

Wild type or RNAi blistered adult *C. elegans* were collected and washed first with M9 buffer and then with 0.1 M cacodylate buffer (pH 7.4). Animals were pelleted, added to 1 ml of 0.8% glutaraldeyde, 0.7% osmium tetroxide, 0.1 M cacodylate pH 7.4 and incubated on ice for 1.5 hours with occasional mixing. The animals were washed with 0.1 M cacodylate buffer, transferred to a glass depression slide and cut in half with a 23 gauge needle. Bisected animals were transferred into a tube containing 1 ml of fresh fixative (0.8% glutaraldehyde, 0.7% osmium tetroxide, 0.1 M cacodylate pH 7.4) and incubated on ice for 2 hrs. After washing with 0.1 M cacodylate buffer, the bisected animals were fixed overnight on ice in 1% osmium tetroxide in 0.1 M cacodylate buffer. Animals were washed several times in 0.1 M cacodylate buffer, dehydrated using graded alcohols through propylene oxide, infiltrated and embedded in Embed-812 (Electron Microscopy Sciences, Ft. Washington, PA). The animals were teased into parallel arrangement with an eyelash probe prior to polymerization at 60°C for 16 hours. Sections (0.5 mm) were evaluated for orientation and ultrasections (800 Å thick)

were collected on 200 mesh copper grids, stained with uranyl acetate and lead citrate, and cross sections were examined with a Philips EM201 electron microscope.

Ultrastructural analysis revealed that cuticle of RNAi Duox animals was grossly abnormal. In normal animals three cuticle layers were seen clearly: the cortical (outer), median and basal (inner) layer, as described previously (Cox, G.N., *et al.*, 1981). The median layer is composed of struts connecting the cortical and basal layers, with a fluid-filled space between these layers. The RNAi animals frequently showed separation between the cortical and the basal layers, with marked expansion of the fluid cavity and broken and distended struts that were still visible on these layers. These separations occurred mainly over bundles of muscle fiber and are likely to account for the formation of the blisters observed using light microscopy. Thus, the cuticle structure was severely affected in RNAi Duox animals.

EXAMPLE 12

Construction of Duox peroxidase domain expression plasmids

The polymerase chain reaction was used to amplify the peroxidase domains of h-Duox (amino acid residues 1-593, SEQ ID NO:31) and Ce-Duox (amino acid residues 1-590, SEQ ID NO:32) from the cloned full length sequences. The primers were designed to introduce an N-terminal BamH I site and a C-terminal Not I site. PCR products were digested with BamH I and Not I and ligated into the pET-32a(+) vector from Novogen (Madison, WI). Plasmids were transformed into BL21(DE3) cells containing the chloramphenicol-resistant plasmid pT-groE (Yasukawa, *et al.*, 1995), which expresses the chaperonins groES and groEL from the T7 promoter. The pT-groE expression vector in BL21(DE3) cells was a generous gift from Dr. Lee-Ho Wang (University of Texas Health Science Center, Houston, TX) and Dr. Shunsuke Ishii (Institute of Physical and Chemical Research, Ibaraki, Japan). LB-agar plates containing both ampicillin and chloramphenicol were used to isolate colonies.

Expression of Duox peroxidase domains

A 0.5 ml LB overnight culture of cells containing plasmid with the peroxidase domain from h-Duox or Ce-Duox was used to inoculate 50 ml of modified TB medium (Sandhu *et al.*, 1993) containing 0.5 mM d-aminolevulinic acid, 100 mg/ml ampicillin and 25 mg/ml chloramphenicol in a 250 ml flask. Bacteria were grown at 37°C in a shaker at 200 RPM until the cell density measured 0.7 OD at 600 nm. Isopropyl-b-D-thiogalactopyranoside (1 mM) was added and the culture was continued at 25°C for 24 hours at 150 RPM. Cells were pelleted at 4,500 x g and resuspended in PBS containing 4-(2-aminoethyl)benzenesufnyl fluoride (2 mM), bestatin (130 nM), *trans*-epoxysuccinyl-L-leucyl-amido(4-guanidino)butane (1.4 nM), leupeptin (1 nM) and aprotinin (0.3 nM). The cell suspension was then sonicated on ice.

Biochemical Activities of the Expressed Peroxidase Domains of Ce-Duox1 and h-Duox1

The peroxidase domains of Ce-Duox (residues 1-590, SEQ ID NO:32) and h-Duox1 (residues 1-593, SEQ ID NO:31) were expressed in *E. coli*, as described above. A lysate from these cells was analyzed for peroxidase activity. The results showed that the lysates from *E. coli*, expressing both the human and the *C. elegans* peroxidase-homology domains from Duox, demonstrated peroxidase activity towards TMB, a well-characterized peroxidase substrate. The activity was inhibited by the peroxidase inhibitor aminobenzohydrazide. Lysates from *E. coli* expressing the peroxidase domains of h-Duox and Ce-Duox, but not those from vector control cells, also catalyzed the cross-linking of tyrosine ethyl ester. Two major fluorescent products were seen, as were also seen in hydrolysates of cuticle protein; peak 1 was identified by co-chromatography with authentic material and mass spectral analysis as dityrosine, while peak 2 was identified as tri-tyrosine by mass spectral analysis as above.

EXAMPLE 13

Activity assays

The 3,3',5,5'-tetramethylbenzidine (TMB) liquid substrate system (Sigma, St. Louis, MO) was used to assay peroxidase activity (Holland *et al.*, 1974). To 1 ml aliquots of the TMB substrate system 100 mg of lysate protein from cells expressing either the human Duox1 peroxidase domain, Ce-Duox1 peroxidase domain or a vector control was added. The peroxidase reactions were performed in triplicate and activity was monitored at 655 nm with a Beckman DU640B spectrophotometer. Some samples contained 30 mM aminobenzoic acid hydrazide, a peroxidase inhibitor (Kettle *et al* 1995).

To assay tyrosine cross-linking, tyrosine ethyl ester (20 mM) was dissolved in 10 ml of PBS buffer supplemented with 80 ml of 3% H_2O_2. To 1 ml aliquots, 100 mg of *E. coli* lysate protein was added, samples were incubated for 1 hour, and the reaction was quenched using an equal volume of 12 M HCl. Samples were analyzed for di- and tri-tyrosine as above.

NADPH-Dependent Superoxide Generation Assay

In one embodiment of the present invention, NIH 3T3 cells stably transfected with the human duox2 gene (SEQ ID NO:1) are analyzed for superoxide generation using the lucigenin (Bis-N-methylacridinium luminescence assay (Sigma, St. Louis, MO, Li et al. (1998) *J. Biol. Chem.* 273, 2015-2023). Cells are washed with cold HANKS' solution and homogenized on ice in HANKS' buffer containing 15% sucrose using a Dounce homogenizer. Cell lysates are frozen immediately in a dry ice/ethanol bath. For the assay, 30 μg of cell lysate is mixed with 200 μM NADPH and 500 μM lucigenin. Luminescence is monitored using a LumiCounter (Packard) at three successive one minute intervals and the highest value is used for comparison. Protein concentration is determined by the Bradford method.

Superoxide generation is monitored in lysates from some of the stably transfected cell lines and was compared with superoxide generation by the untransfected NIH 3T3 cell lysates. The results are show that the transfected cells possess the highest degree of morphological changes by microscopic examination corresponding to

the highest degree of superoxide generation. The luminescent signal is inhibited by superoxide dismutase and the general flavoprotein inhibitor diphenylene iodonium, but is unaffected by added recombinant human p47phox, p67phox and Rac1(GTP-γS), which are essential cytosolic factors for the phagocyte respiratory-burst oxidase.

In an alternate embodiment of the present invention, cells that are stably transfected with hduox2 (YA28) or with empty vector (NEF2) are grown in 10 cm tissue culture plates in medium containing DMEM, 10% calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 1 μg/ml puromycin to approximately 80% confluency. Cells (five tissue culture plates of each cell type) are washed briefly with 5 ml phosphate buffered saline (PBS) then dissociated from the plates with PBS containing 5 mM EDTA. Cells are pelleted by centrifuging briefly at 1000 x g.

To permeabilize the cells, freeze thaw lysis is carried out followed by passage of the cell material through a small bore needle. The supernatant is removed and the cells frozen on dry ice for 15 minutes. After cells thaw, 200 μl lysis buffer (HANKS' Buffered Salt Solution - HBBS) containing a mixture of protease inhibitors from Sigma (Catalog # P2714) is added. Cells on ice are passed through an 18 guage needle 10 times and 200 μl of HBSS buffer containing 34% sucrose was added to yield a final concentration of 17% sucrose. Sucrose appears to enhance stability upon storage. The combination of freeze-thawing and passage through a needle results in lysis of essentially all of the cells, and this material is referred to as the "cell lysate."

The cell lysates are assayed for protein concentration using the BioRad protein assay system. Cell lysates are assayed for NADPH-dependent chemiluminescence by combining HBSS buffer, arachidonic acid, and 0.01 – 1 μg protein in assay plates (96 well plastic plates). The reaction is initiated by adding 1.5 mM NADPH and 75 μM lucigenin to the assay mix to give a final concentration of 200 μM NADPH and 10 μM lucigenin, and the chemiluminescence is monitored immediately. The final assay volume is about 150 μl. The optimal arachidonic acid concentration is between about 50-100 μM. A Packard Lumicount luminometer is used to measure

chemiluminescence of the reaction between lucigenin and superoxide at 37°C. The plate is monitored continuously for 60 minutes and the maximal relative luminescence unit (RLU) value for each sample is plotted. Results show that the presence of NaCl or KCl within a concentration range of 50-150 μM is important for optimal activity. $MgCl_2$ (1-5 mM) further enhances activity by about 2-fold. This cell-free assay for duox2 NADPH-oxidase activity is useful for screening modulators (inhibitors or stimulators) of the duox2 enzyme. The assay may also be used to detect and duox NADPH-oxidase activity in general and to screen for modulators (inhibitors or stimulators) of the duox family of enzymes.

Nitro Blue Tetrazolium Reduction by Superoxide Generated by NIH 3T3 cells Transfected with the Duox2 cDNA (SEQ ID NO:1)

Superoxide generation by intact cells is monitored by using superoxide dismutase-sensitive reduction of nitroblue tetrazolium. NEF2 (vector alone control), YA26 (duox2 (SEQ ID NO:1)-transfected) and YA28 (duox2 (SEQ ID NO:1)-transfected) cells are plated in six well plates at 500,000 cells per well. About 24 hours later, medium is removed from cells and the cells are washed once with 1 mL Hanks solution (Sigma, St. Louis, MO). About 1 mL of filtered 0.25% Nitro blue tetrazolium (NBT, Sigma) is added in Hanks without or with 600 units of superoxide dismutase (Sigma) and cells are incubated at 37°C in the presence of 5% CO_2. After 8 minutes the cells are scraped and pelleted at more than 10,000g. The pellet is re-suspended in 1 mL of pyridine (Sigma) and heated for 10 minutes at 100°C to solubilize the reduced NBT. The concentration of reduced NBT is determined by measuring the absorbance at 510 nm, using an extinction coefficient of 11,000 $M^{-1}cm^{-1}$. Some wells are untreated and used to determine cell number.

The data indicate that the duox2 (SEQ ID NO:1)-transfected cells generated significant quantities of superoxide. Because superoxide dismutase is not likely to penetrate cells, superoxide must be generated extracellularly. The amount of superoxide generated by these cells is about 5-10% of that generated by activated human neutrophils.

EXAMPLE 14

Modification of Intracellular Components in Duox2 Transfected Cells

To test whether superoxide generated by duox2 can affect intracellular "targets", aconitase activity in control and duox-transfected cell lines is monitored using methods as described in Suh et al. (1999) *Nature* 401, 79-82. Aconitase contains a four-iron-sulphur cluster that is highly susceptible to modification by superoxide, resulting in a loss of activity, and has been used as a reporter of intra-cellular superoxide generation. Acotinase activity is determined as described in Gardner et al. (1995) *J. Biol. Chem.* 270, 13399-13405. Acotinase activity is significantly diminished in all three duox-transfected cell lines designated YA26, YA28 and YA212 as compared to the transfected control. Approximately 50% of the aconitase in these cells is mitochondrial, based on differential centrifugation, and the cytosolic and mitochondrial forms are both affected. Control cytosolic and mitochondrial enzymes that do not contain iron-sulfur centres are not affected. Superoxide generated in duox2-transfected cells is therefore capable of reacting with and modifying intracellular components.

EXAMPLE 15

Tumor Generation in Nude Mice Receiving Cells Transfected with the Human duox2 cDNA (SEQ ID NO:1)

About 2 x 10^6 NIH 3T3 cells (either hduox2-transfected with SEQ ID NO:1 or cells transfected using empty vector) are injected subdermally into the lateral aspect of the neck of 4-5 week old nude mice. Three to six mice are injected for each of three duox1-transfected cell lines, and 3 mice are injected with the cells transfected with empty vector (control). After 2 to 3 weeks, mice are sacrificed. The tumors are fixed in 10% formalin and characterized by histological analysis. Tumors averaged 1.5 x 1 x 1 cm in size and show histology typical of sarcoma type tumors. In addition, tumors appear to be highly vascularized with superficial capillaries. Eleven of twelve mice injected with duox2 gene-

transfected cells develop tumors, while none of the three control animals develop tumors.

In another study, 15 mice are injected with duox2-transfected NIH 3T3 cells. Of the 15 mice injected, 14 show large tumors within 17 days of injection, and tumors show expression of duox1 mRNA. Histologically, the tumors resemble fibrosarcomas and are similar to ras-induced tumors. Thus, ras and duox2 are similarly potent in their ability to induce tumorigenicity of NIH 3T3 cells in athymic mice.

EXAMPLE 16

Demonstration of the Role of Duox2 in Non-Cancerous Growth

A role in normal growth is demonstrated in rat aortic vascular smooth-muscle cells by using antisense to rat duox2. Transfection with the antisense DNA results in a decrease in both superoxide generation and serum-dependent growth. Duox2 is therefore implicated in normal growth in this cell type.

All patents, publications and abstracts cited above are incorporated herein by reference in their entirety. It should be understood that the foregoing relates only to preferred embodiments of the present invention and that numerous modifications or alterations may be made therein without departing from the spirit and the scope of the present invention as defined in the following claims.

CLAIMS

We claim,

1. A protein capable of stimulating superoxide production or generating peroxidative reactions, wherein the protein comprises the amino acid sequence of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:31, or SEQ ID NO:32, a fragment thereof, or a conservative substitution thereof, wherein the protein, the fragment thereof or the conservative substitution thereof is capable of generating reactive oxygen intermediates or generating peroxidative reactions.

2. A protein capable of stimulating superoxide production or generating peroxidative reactions, wherein the protein comprises the amino acid sequence of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:31, or SEQ ID NO:32, a fragment thereof or a conservative substitution thereof.

3. A protein capable of stimulating superoxide production or generating peroxidative reactions, wherein the protein comprises the amino acid sequence of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:31, or SEQ ID NO:32, a deletion thereof or an addition thereto of no more than about 5% of the amino acid sequence, or a conservative substitution thereof, wherein the conservative substitution comprises substitution of

a) alanine, serine, or threonine for each other;
b) aspartic acid or glutamic acid for each other;
c) asparagine or glutamine for each other;
d) arginine or lysine for each other;
e) isoleucine, leucine, methionine, or valine for each other; and
f) phenylalanine, tyrosine, or tryptophan for each other.

4. A protein comprising the amino acid sequence of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:31, or SEQ ID NO:32.

5. A nucleotide sequence encoding for the protein, the fragment thereof or the conservative substitution thereof, as recited in any one of Claims 1 to 4.

6. The nucleotide sequence of Claim 5, wherein the nucleotide sequence comprises SEQ ID NO:1 or SEQ ID NO:3, a fragment thereof or a conservative substitution thereof.

7. A nucleotide sequence comprising SEQ ID NO:1 or SEQ ID NO:3.

8. A vector, wherein the vector comprises a nucleotide sequence encoding for the protein, the fragment thereof or the conservative substitution thereof, as recited in any one of Claims 1 to 4.

9. The vector of Claim 8 wherein the nucleotide sequence comprises SEQ ID NO:1 or SEQ ID NO:3, a fragment thereof or a conservative substitution thereof.

10. A vector, wherein the vector comprises a nucleotide sequence comprising SEQ ID NO:1 or SEQ ID NO:3, a fragment thereof or a conservative substitution thereof.

11. A cell containing the vector of Claim 8.

12. A cell containing the vector of Claim 10.

13. An antibody generated against the protein of any one of Claims 1 to 4.

14. An antibody generated against the amino acid sequence of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:31, or SEQ ID NO:32.

15. A method of stimulating superoxide formation or generating peroxidative reactions, comprising administration, in vitro or in vivo, of a composition comprising the protein of any one of Claims 1 to 4 in a pharmaceutically acceptable carrier.

16. A method of stimulating superoxide formation or generating peroxidative reactions, comprising administration, in vitro or in vivo, of a composition comprising the protein of Claim 4 in a pharmaceutically acceptable carrier.

17. A method of stimulating superoxide formation or generating peroxidative reactions, comprising administration, in vitro or in vivo, of a composition comprising the vector of Claim 8 in a pharmaceutically acceptable carrier.

18. A method of stimulating superoxide formation or generating peroxidative reactions, comprising administration, in vitro or in vivo, of a composition comprising the vector of Claim 9 in a pharmaceutically acceptable carrier.

19. A method for determining activity of a drug or a chemical comprising measuring enzymatic activity of the protein, the fragment thereof or the conservative substitution thereof, as recited in any one of Claims 1 to 4, to stimulate superoxide production or generate peroxidative reactions following administration of the drug or the chemical.

20. A method for determining activity of a drug or a chemical comprising measuring enzymatic activity of the protein recited in Claim 4 to stimulate superoxide production or generate peroxidative reactions following administration of the drug or the chemical.

21. The method of Claim 19, wherein the enzymatic activity is NADPH- dependent or NADH-dependent superoxide generation, tetramethylbenzidine oxidation or tyrosine cross-linking.

22. The method of Claim 19, wherein the enzymatic activity is NADPH- dependent or NADH-dependent diaphorase activity, tetramethylbenzidine oxidation or tyrosine cross-linking.

23. A method for determining activity of a drug or a chemical comprising measuring binding of the drug or the chemical to the protein, the fragment thereof or the conservative substitution thereof, as recited in any one of Claims 1 to 4.

24. A method for determining activity of a drug or a chemical comprising measuring binding of the drug or the chemical to the protein recited in Claim 4.

25. A method for determining activity of a drug or a chemical comprising measuring the activity of the drug or the chemical to modulate proliferative activity or peroxidative activity of the protein, the fragment thereof or the conservative substitution thereof, as recited in any one of Claims 1 to 4.

26. A method for determining activity of a drug or a chemical comprising measuring the activity of the drug or the chemical to modulate proliferative activity or peroxidative activity of the protein recited in Claim 4.

27. The method of Claim 19, wherein the enzymatic activity is assessed using intact cells, transfected cells or cell lysates thereof.

28. The method of Claim 20, wherein the enzymatic activity is assessed using intact cells, transfected cells or cell lysates thereof.

29. A method for affecting cuticle biogenesis comprising administration to an organism with a cuticle of a composition which affects an activity of a protein comprising duox, a fragment thereof or a conservative substitution thereof, wherein the activity is cuticle biogenesis.

30. The method of Claim 29, wherein the protein comprises the amino acid sequence of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:31, or SEQ ID NO:32, a fragment thereof, or a conservative substitution thereof.

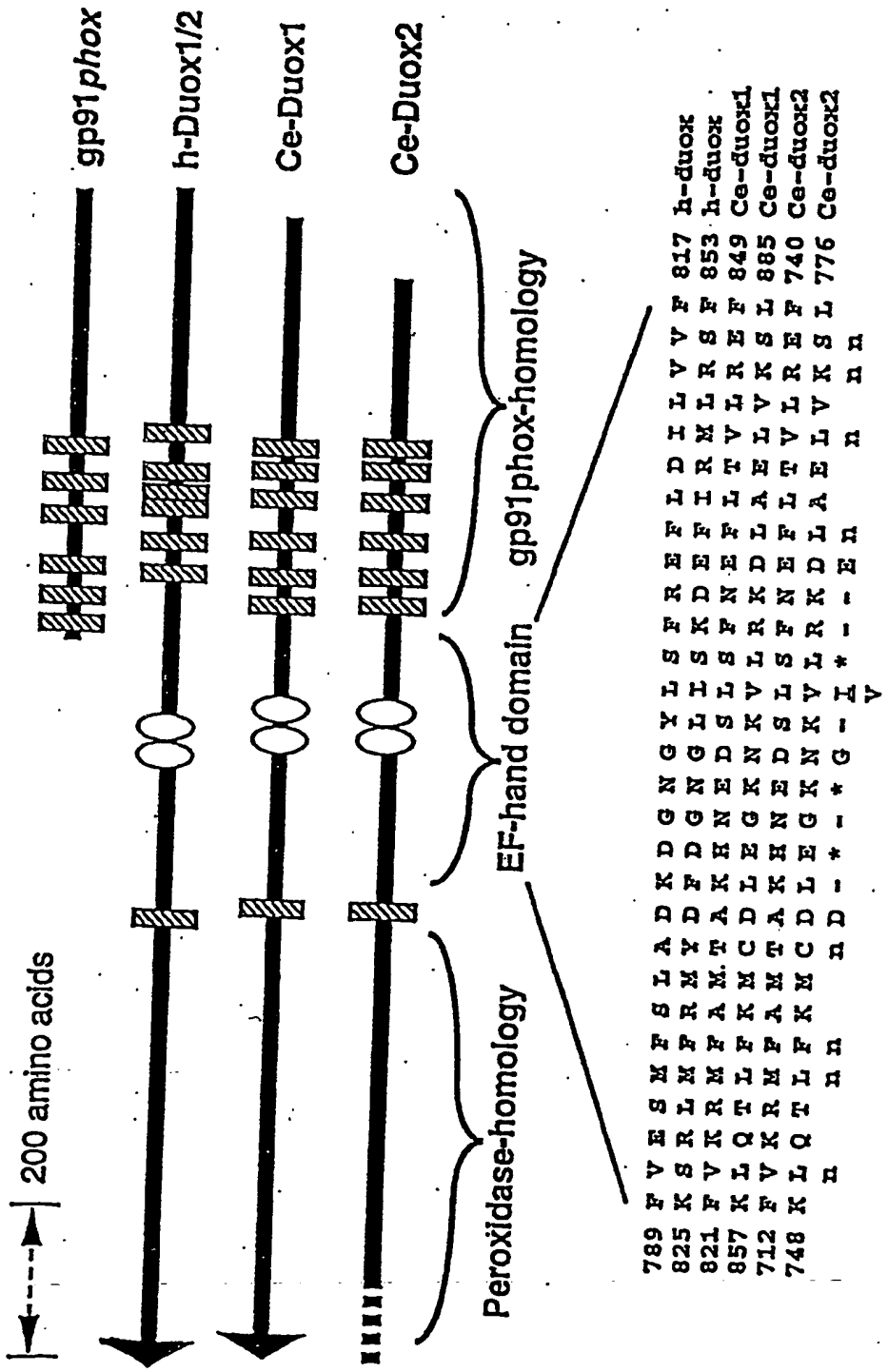
31. Use of a protein comprising duox, a fragment thereof or a conservative substitution thereof for evaluating a biological activity of a drug or a chemical, comprising measuring enzymatic activity of the duox, the fragment thereof or the conservative substitution thereof, to stimulate superoxide production or generate peroxidative reactions following administration of the drug or the chemical.

32. The use of Claim 31, wherein the duox protein comprises the amino acid sequence of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:31, or SEQ ID NO:32, a fragment thereof, or a conservative substitution thereof.

33. The use of Claim 31, wherein the biological activity is cuticle biogenesis, thyroid hormone biosynthesis or fibrosis.

34. The use of Claim 33, wherein the fibrosis is lung fibrosis.

FIGURE 1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Ce-Duox2
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Peroxidase-homology
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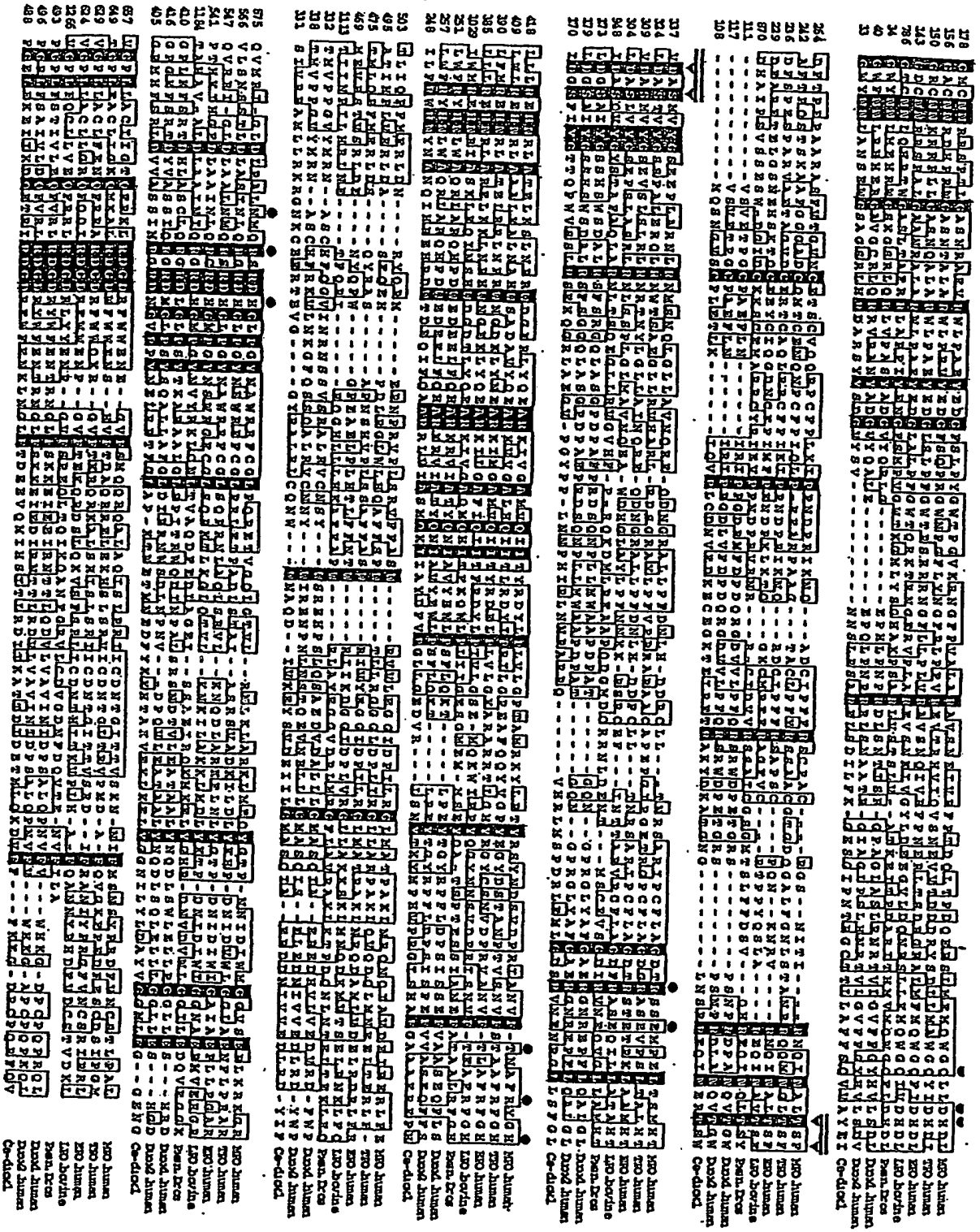


FIGURE 2A

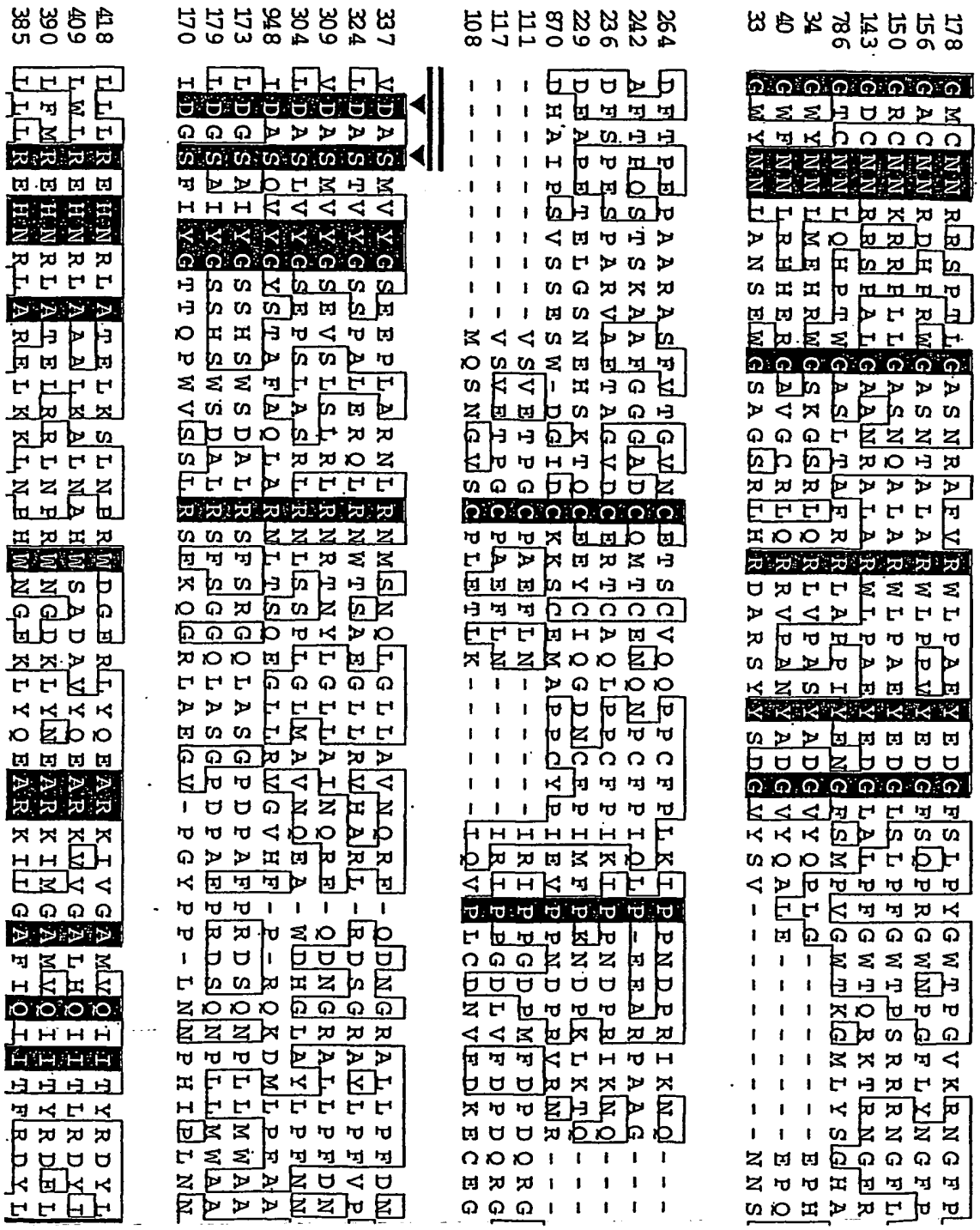
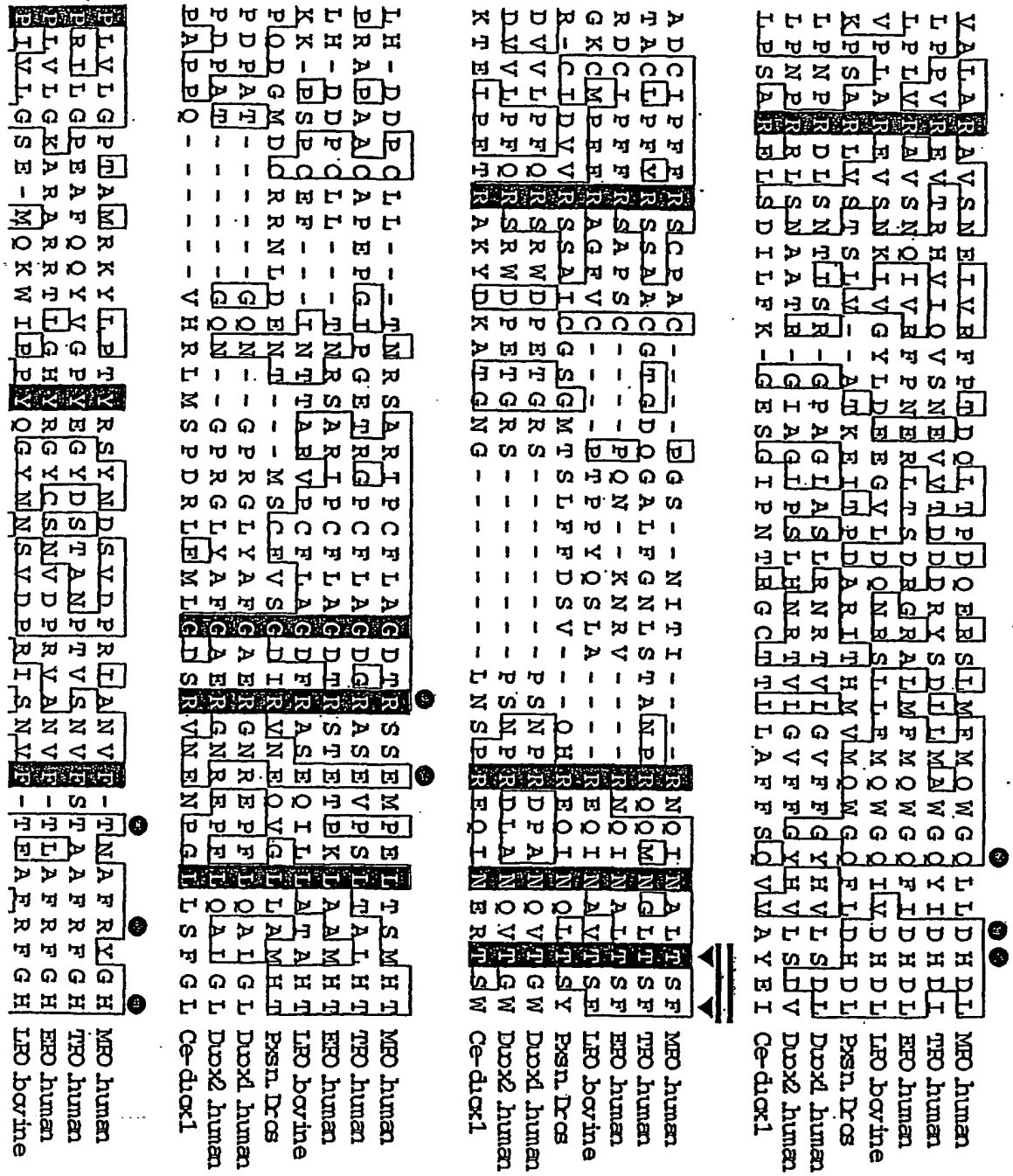


FIGURE 2A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LEO bovine
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Ce-duox1

FIGURE 2A

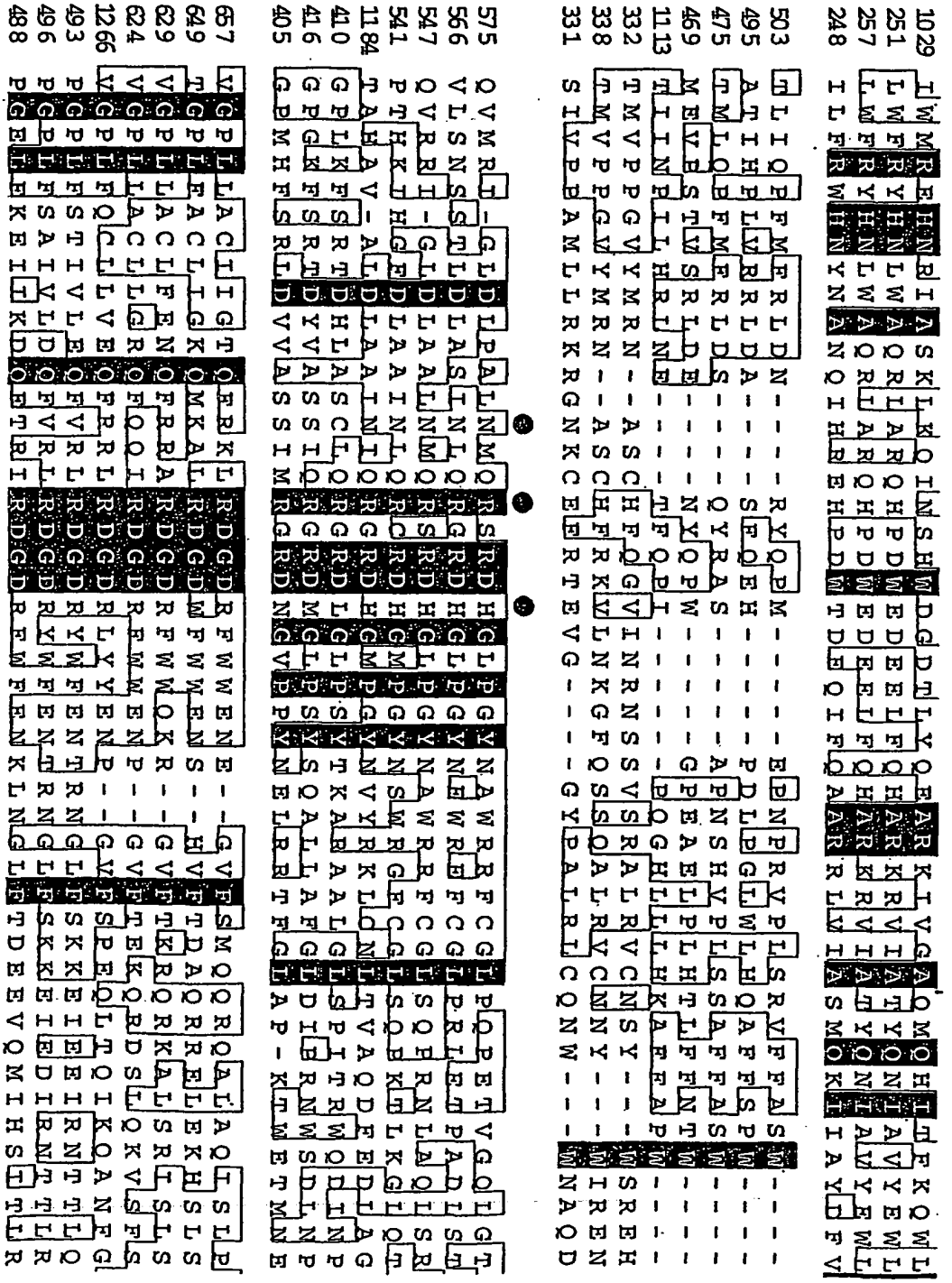
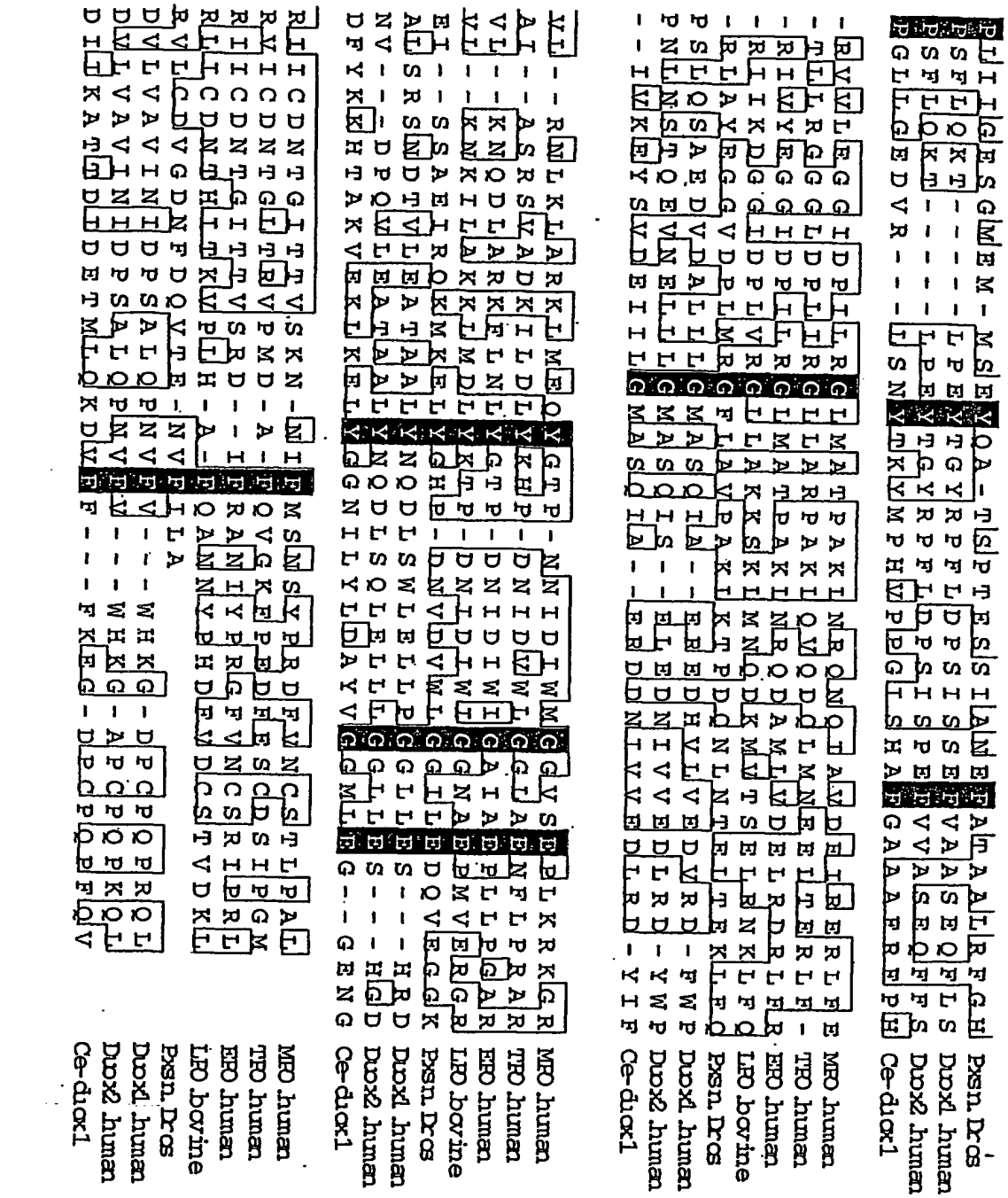


FIGURE 2A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Duox2.human
Ce-duox1
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TPO.human
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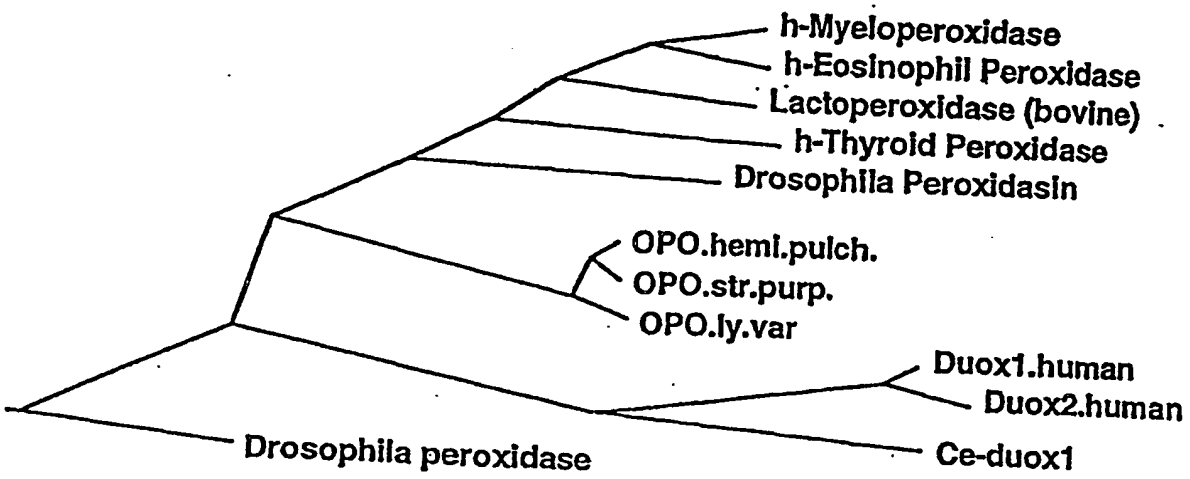
Duox1.human
Duox2.human
Ce-duox1

FIGURE 2A

FIGURE 2B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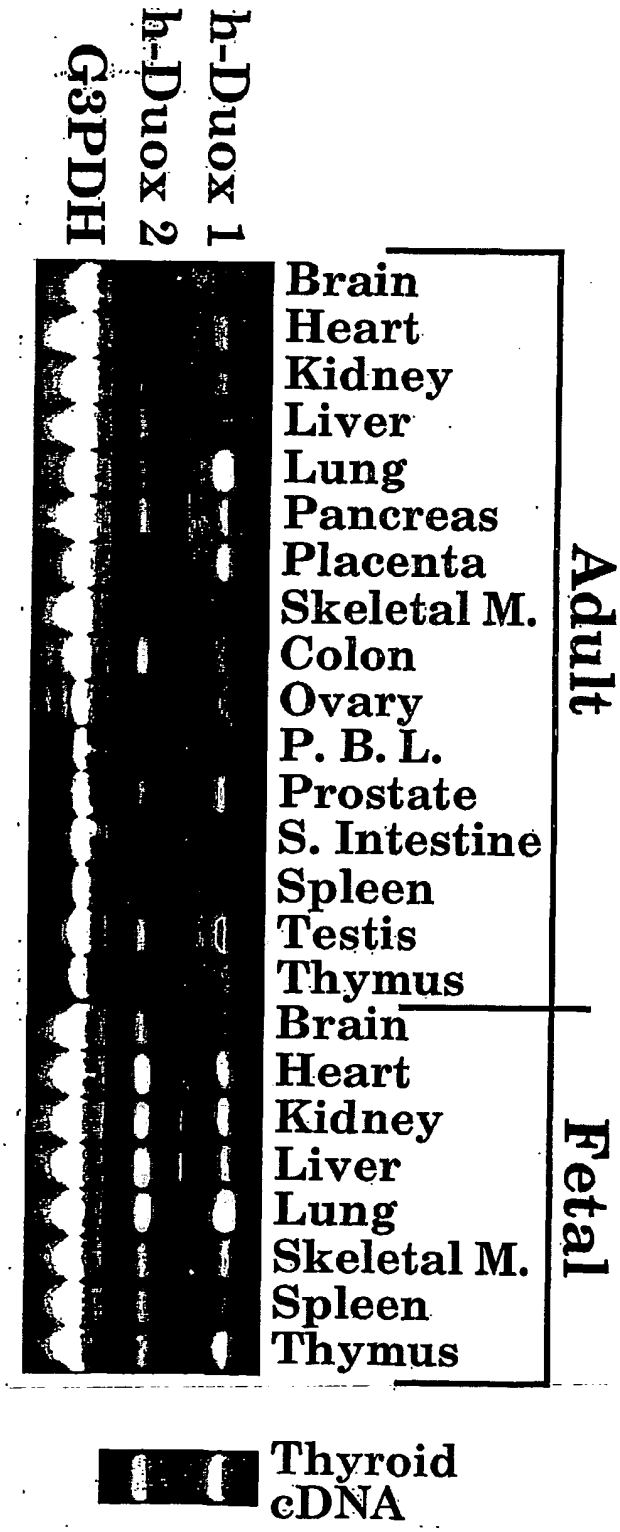
Drosophila peroxidase
Ce-duox1

FIGURE 3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Pancreas
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P. B. L.
Prostate
S. Intestine
Spleen
Testis
Thymus
Adult
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Kidney
Liver
Lung
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Spleen
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Fetal
Thyroid cDNA

SEQ ID NO:1

Human Duox 2 nucleotide sequence

BASE COUNT 1389 a 1873 c 1692 g 1421 t

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4321 agtggcataa atttgaggtg tcagtgttgg tgggaggggg cattggggtc accccctttg
4381 cctccatcct caaagacctg gtcttcaagt catccttggg cagccaaatg ctgtgtaaga
4441 agatctactt catctgggtg acacggaccc agcgtcagtt tgagtggctg gctgacatca
4501 tccaagaggt ggaggagaac gaccaccagg acctggtgtc tgtgcacatt tatgtcaccc
4561 agctggctga gaagttcgac ctcaggacca ccatgctata catctgcgag cggcacttcc
4621 agaaagtgct gaaccggagt ctgttcacgg gcctgcgctc catcacccac tttggccgtc
4681 ccccttcga gcccttcttc aactccctgc aggaggtcca cccacaggtg cgcaagatcg
4741 gggtgttcag ctgcggccct ccaggaatga ccaagaatgt agagaaggcc tgtcagctcg
4801 tcaacaggca ggaccgagcc cacttcatgc accactatga gaacttctga gcctgtcctc
4861 cctggctgct gcttccagta tcctgccttc tcttctgtgc acctaagttg cccagccctg
4921 ctggcaatct ctccatcaga atccacctta ggcctcagct ggagggctgc agagcccctc
4981 ccaatattgg gagaatattg acccagacaa ttatacaaat gagaaaaggc aggagactat
5041 gttctacaat tgcagtgcat gatgattata agtccacctg tttatcaacg gcaccattcc
5101 tgcagccctc cagacttcct gcccttagca agtgcgcaac cagtcaggat ctcccaaaga
5161 agataaagac cactcctcac cccagctcaa gccatggcag gcgtggcaag caaagtgggg
5221 aggagacagt ccctgcttgt gacaagtgtg gaggtgaaaa ggtcaatagt gcttgtctcc
5281 gatagctccc cacatctcta attgacttcc acaaaatcga tgcgttgctt tggtatttgc
5341 ttggactgac atttgaggga ggaggaggct gggatcctct ggctgagaat ctcctcagag
5401 cccagtgcag aagctgtgat gcttagaacc tggacagccc gactgcctca actctgtctc
5461 caggtctatt ccctccagct ccaaaaggag cagccctact tctacccctt cccgtcccca
5521 aagtgtcagc aactttgagg agggcaccag gaaacaaaga tgccttccca gccctgatat
5581 tcttgatgtc accagtgata cccactgccc tgacccctgg gcaggccccc ctctgcatct
5641 actggagtgg tccctgggct cttggggctg aaggattcca gcctctctgc cagatattca
5701 gtactcgatc tcaattcccc tcttccacaa gagttgggtg accagctgtc ctagtttgcc
5761 caggactctc cctgttttag cactgaaagt ctcttgcccc aggaaacccc atcagtccca
5821 ggcagattgg gacagctggt caccttacgc aagagccagg ctgaaacatc ccctccatac
5881 tcagctcttt aacttttctt ttccttttc atcgggctct ttcctaaaaa gctgagctgt
5941 aaaatatttt acatcgaggt ataataaata atcatgtaca tgttttacca ccacccaggt
6001 caagacatag aatgtttcaa catttccatc accccagaaa ctccccttgt acccccttcc
6061 cttcgtctcc cctagctcct agaagcaacc actgatgtga tttctaccaa atccagtttt
6121 ggtcctacta aatatactct tttgagactg gcctctttta ctcaccataa tgcctttgta
6181 attcatccat gctgttgtgt gtatcagcag tttgttcctt ttcattgctg agtagtattc
6241 tattgtagag atgtaccaca gtttgtttat tcttctgttg atggacgttt gggttgtttc

6301 taattttgaa tgattataaa taaaaattct gtgagtgttc ttgtaaaaaa aaaaaaaaaa
6361 aaaaaaaaaa aaaaa

SEQ ID NO:2
Human Duox 2 amino acid sequence (single letter code)

1 mlrarpealm llgalltgsl gpsgsqdals lpwevqrydg wfnnlrhher gavgcrlqrr
61 vpanyadgvy qaleepqlpn prrlsnaatr glaglpslhn rtvlgvffgy hvlsdvvsve
121 tpgcpaefln irippgdlvf dpdqrgdvvl pfqrsrwdpe tgrspsnprd lanqvtgwld
181 gsalygsshs wsdalrsfsg gqlasgpdpa fprdsqnpll mwaapdpatg qngprglyaf
241 gaergnrepf lqalgllwfr yhnlwaqrla rqhpdwedee lfqharkrvl atyqniavye
301 wlpsflqktl peytgyrpfl dpslspefvv aseqffstmv ppgvymmas chfrkvlnkg
361 fqssqalrvc nnywirenpn lnstqevnel llgmasqise lednivvedl rdywpgpgkf
421 srtdyvassl qrgrdmglps ysqallafgl dlpmwsdln pnvdpqvlea taalynqdls
481 qlelllggll eshgdpgplf saivldqfvr lrdgdrywfe ntmglfskk eiedlmttl
541 rdvlvavinl dpsalqpnvf vwhkgapcpq pkqlttdglp qcapltvldf fegsspgfai
601 tiialcclpl vsllisgvva yfrgrehkkl qkklkesvkk eaakdgvpam ewpgpkerss
661 plllqllsdr clqvlnrhlt vlrvvqlqpl qqvnlilsnn rgcrtlilki pkeydlvllf
721 sseeergafv qqlwdfcvrw alglhvaems ekelfrkavt kqqrerllel ffrhlfaqvl
781 dinqadagtl pldssqkvre altcelsrae faeslglkpq dmfvesmfsl adkdgngyls
841 frefldilvv fmkgspedks rlmftmydld engflskdef ftmmrsflei snnclskaql
901 aevvesmfre sgfqdkeelt wedfhfmlrd hdselrftql cvkggggggn girdlfkqnl
961 scrvsfitrt pgershpqgl gppvpeapel ggpglkkrfg kkaavptprl ytealqekmq
1021 rgflaqklqq ykrfvenyrr hivcvaifsa icvgvfadra yyygfalpps diaqttlvgl
1081 ilsrgtaasv sfmfsyillt mcrnlitflr etflnryvpf daavdfhrwi amaavvlail
1141 hsaghavnvy ifsvsplsll acifpnvfvn dgsklpqkfy wwffqtvpgm tgvllllvla
1201 lmyvfashhf rrrsfrgfwl thhlyillya lilihgsyal iqlptfhiyf lvpaliyggd
1261 klvslsrkkv eisvvkaell psgvtylqfq rpqgfeyksg qwvriaclal gtteyhpftl
1321 tsaphedtls lhiravgpwt trlrelyssp kgngcagypk lyldgpfgeg hqewhkfevs
1381 vlvgggigvt pfasilkdlv fkssigsqml ckkiyfiwvt rtqrqfewla dllqeveend
1441 hqdlvsvhly vtqlaekfdl rttmlyicer hfqkvlnrsl ftglrsithf grppfepffn
1501 slqevhpqvr kigvfscgpp gmtknvekac qlvnrqdrah fmhhyenf

SEQ ID NO:3
Ce. Duox 1 nucleotide sequence
BASE COUNT 1340 a 891 c 983 g 1280 t
ORIGIN

1 atgcgctcaa aacatgtgct gtacatagct atactgttca gttcaatttt tggagggaaa
61 ggaatccaac aaaatgagga atttcaaaga tacgacggat ggtacaacaa tctggcgaat
121 agtgaatggg gttctgctgg aagtcggctg catagagatg cacgttccta ctactcagac
181 ggtgtatatt cagtgaataa ctcacttccg tccgcccgtg aactctccga tatactattc
241 aaaggagagt ccggtatacc taatacaaga ggatgcacga ctttattggc atttttcagt
301 caagtagttg ctcatgaaat aatgcaatca aatggagtat cctgtccact agagacactt
361 aaaattcaag tacccctatg tgataatgta tttgataaag aatgtgaggg aaagacagaa
421 atcccattta cacgtgccaa atacgataaa gcaactggaa atgggctcaa ctcacctcga
481 gaacaaatca atgaacggac ttcatggatt gatggatcat tcatctatgg taccacccag
541 ccatgggtgt cctcattaag atctttcaaa caagggcggt tggctgaagg tgtacctgga
601 tatccaccac ttaacaaccc acatattcca ttgaataacc ccgctccgcc acaagtacat
661 cgattgatga gtccagatag attatttatg ttgggagact cgcgtgtgaa tgagaatcca
721 ggtcttctct catttggtct gatcctcttc cgttggcata actacaatgc aaatcaaatc
781 catcgagaac atcctgactg gacagacgaa caaatcttcc aggcagcacg tcgtttggtg
841 attgcatcta tgcagaagat tattgcatat gactttgttc cagggctgtt aggtgaagac
901 gttcgtttgt caaactacac caaatacatg ccacatgttc cacctggaat ctcgcatgct
961 tttggagcag ccgccttcag gttccctcac tcaattgtgc caccagcaat gcttctgaga
1021 aaacgaggaa ataaatgtga attccggacg gaagttggtg gatatcctgc attgagattg
1081 tgccagaatt ggtggaatgc gcaggatatt gtaaaggagt acagtgtgga tgagattatt
1141 cttggaatgg caagccagat agctgaacga gatgataaca tagtagttga agatcttcgt
1201 gattacatct tcggaccaat gcatttctct cgtttggatg ttgttgcttc atcaataatg
1261 agaggaaggg acaatggagt accaccgtat aatgaattga gaagaacatt cggacttgcg
1321 ccaaagacat gggagacaat gaatgaagac ttttacaaga agcatactgc aaaggtggag
1381 aagttgaaag agttgtatgg aggcaatatt ttatatttgg atgcttatgt aggaggaatg
1441 ctggaaggag gtgaaaatgg gcctggagag ttgttcaaag aaatcataaa ggatcaattc
1501 acccgtattc gtgatggaga tagattctgg tttgagaata aattgaatgg attattcact
1561 gatgaagaag ttcaaatgat tcatagtatt acacttcgag atattatcaa agcaaccacc
1621 gatatcgatg agacaatgct tcagaaggat gtattcttct tcaaggaagg tgacccgtgc
1681 ccgcaaccat tccaagtgaa cacaactgga cttgaaccat gtgttccatt tatgcaatca
1741 acttattgga ctgataatga caccacttat gttttcaccc taattggatt agcatgtgtg
1801 ccattaattt gctatggaat tggccgatac ttggttaatc gtcgcattgc tattggccac
1861 aacagtgctt gtgacagcct aactactgac tttgcaaatg atgattgtgg cgcgaaggga
1921 gatatttatg gtgtaaatgc tttggaatgg cttcaagaag agtacatacg acaggtcagg
1981 atagaaatag aaaacaccac gttggcagta aagaagccac gcggtggaat ccttcgaaaa
2041 attcgttttg aaactggaca gaagattgag ttattccact ctatgccgaa tccatcagca
2101 atgcacggac catttgtact tctgtctcaa aagaataatc atcatttggt gataagattg

2161 tcgtctgata gagatttatc taaatttttg gatcaaatta gacaggcggc tagtggaatc
2221 aatgcagagg ttatcataaa ggatgaggag aattctattc tcttatccca agcaatcaca
2281 aaagaacgcc gtcaagaccg actggacctg ttcttccgtg aagcctacgc aaaagcattc
2341 aatgatagtg aacttcaaga ttcggaaact tcatttgact catcaaatga tgatatatta
2401 aatgagacaa tatctcgtga ggaactggca agtgcaatgg gaatgaaagc gaataatgag
2461 tttgtgaaga gaatgttcgc gatgattgca aaacataatg aggattcgct cagtttcaat
2521 gagtttttga cagtcttgag agagtttgtt aatgctcctc aaaagcaaaa actgcaaact
2581 ctattcaaaa tgtgtgattt ggagggaaag aacaaggtac tccgaaagga tctcgcggaa
2641 ctcgtcaagt ccctcaatca aaccgctgga gttcacatta ctgaaagtgt gcagcttcga
2701 ttattcaatg aagtgttgca ctatgcagga gtgagcaatg atgccaagta cctgacttac
2761 gacgatttca atgctctgtt ctcggatata cctgacaagc aaccagttgg actgccgttc
2821 aatcgaaaga actatcagcc aagtattgga gaaacatctt ctctgaactc atttgccgtc
2881 gtggatcgat ccatcaacag ttcagcaccg ctaactttga tccacaaagt ttcagcgttc
2941 ttggagacct atcgccaaca cgttttcatt gtcttctgct ttgttgccat caatcttgtt
3001 cttttcttcg aacggttttg gcattatcgt tacatggcgg aaaacaggga tctccgacga
3061 gtaatgggag ctggaatcgc tattactcgt ggtgccgcgg gagccttgtc attttgcatg
3121 gcgttgatat tgctgacagt ttgtagaaac ataatcacac ttcttcgaga gacagtcatt
3181 gcgcagtata ttccatttga ctcggctatt gcgttccaca agatcgttgc gctctttgcg
3241 gctttctggg ccactcttca caccgttgga cattgtgtca atttctatca cgttggaact
3301 caaagtcaag aaggtcttgc ttgtctcttt caggaagcat tctttggatc caacttcctt
3361 ccttcaatca gttactggtt cttcagcaca attacaggtc tgacaggaat tgcattggtc
3421 gctgtcatgt gcatcattta tgtttttcgcg ttaccatgtt tcattaagag agcttatcac
3481 gcattccggc tcacacatct tctcaatatt gccttttacg cacttactct tcttcatggg
3541 cttccaaagt tgttggattc tcccaaattt ggctactacg ttgttggtcc catcgtgtta
3601 tttgtaattg atcgcataat tggtttgatg caatattaca aaaaattaga aattgtaaac
3661 gcagaaatcc ttccatcaga tattatatac atcgagtacc gtcgtccaag agagtttaaa
3721 tataaatcag gacaatgggt tactgtatca tcaccatcaa tatcatgtac ctttaatgaa
3781 tctcacgcat tctcgattgc ctcaagtcca caggatgaga atatgaagtt gtatataaaa
3841 gcagttggac catggacatg gaagttgaga agcgaattga taagatcatt gaatacagga
3901 tcgccatttc cattaatcca tatgaaagga ccatatggtg atggtaacca agaatggatg
3961 gattatgaag ttgcaataat ggttggagca ggaatcggag tgactccata tgcatcgaca
4021 cttgttgatc ttgtacaacg aacatcaagt gactcatttc acagagttcg ttgccgtaaa
4081 gtatatttcc tatgggtgtg ctcaactcac aagaactatg aatggtttgt ggatgtgctc
4141 aagaacgtgg aagaccaagc aaggtcggga attttggaga cacatatctt tgtcactcag
4201 acgttccaca agtttgattt gagaactact atgctttaca tttgcgagaa gcacttccgt
4261 gccaccaact caggaatttc aatgtttact ggtctccacg ctaagaacca tttcggacgg

4321 cccaacttca aagctttctt ccaatttatt cagagtgaac ataaggagca atccaaaatc
4381 ggagtgttca gttgtggacc tgtaaacttg aatgaaagta tagctgaagg atgtgcagat
4441 gccaaccgac aacgagatgc tccttcattt gcacatcgct ttgaaacgtt ctaa

SEQ ID NO:4
Ce. Duox 1 amino acid sequence (single letter code)

1 mrskhvlyla llfsslfggk giqqneefqr ydgwynnlan sewgsagsrl hrdarsyysd
61 gvysvnnslp sarelsdilf kgesgipntr gcttllaffs qvvaheimqs ngvscpletl
121 kiqvplcdnv fdkecegkte ipftrakydk atgnglnspr eqinertswi dgsfiygttq
181 pwvsslrsfk qgrlaegvpg ypplnnphip lnnpappqvh rlmspdrlfm lgdsrvnenp
241 gllsfglllf rwhnynanql hrehpdwtde qlfqaarrlv lasmqkllay dfvpgllged
301 vrlsnytkym phvppgisha fgaaafrfph sivppamllr krgnkcefrt evggypalrl
361 cqnwwnaqdi vkeysvdeii lgmasqlaer ddnivvedlr dyifgpmhfs rldvvassim
421 rgrdngvppy nelrrtfgla pktwetmned fykkhtakve klkelyggni lyldayvggm
481 leggengpge lfkeiikdqf trirdgdrfw fenklnglft deevqmlhsi tlrdiikatt
541 didetmlqkd vfffkegdpc pqpfqvnttg lepcvpfmqs tywtdndtty vftllglacv
601 plicygigry lvnrriaigh nsacdslttd fanddcgakg diygvnalew lqeeyirqvr
661 lelenttlav kkprgglirk irfetgqkle lfhsmpnpsa mhgpfvllsq knnhhlvirl
721 ssdrdlskfl dqirqaasgi naevlikdee nsillsqait kerrqdrldl ffreayakaf
781 ndselqdset sfdssnddil netisreela samgmkanne fvkrmfamia khnedslsfn
841 efitvlrefv napqkqklqt lfkmcdlegk nkvlrkdlae lvkslnqtag vhitesvqlr
901 lfnevlhyag vsndakylty ddfnalfsdi pdkqpvglpf nrknyqpsig etsslnsfav
961 vdrslnssap ltlihkvsaf letyrqhvfi vfcfvalnlv lfferfwhyr ymaenrdlrr
1021 vmgaglaitr gaagalsfcm alilltvcrn litllretvi aqyipfdsai afhklvalfa
1081 afwatlhtvg hcvnfyhvgt qsqeglaclf qeaffgsnfl psisywffst itgltglalv
1141 avmcliyvfa lpcfikrayh afrlthllnl afyaltllhg lpklldspkf gyyvvgpivl
1201 fvidriiglm qyykklelvn aeilpsdiiy ieyrrprefk yksgqwvtvs spsisctfne
1261 shafslassp qdenmklyik avgpwtwklr selirslntg spfplihmkg pygdgnqewm
1321 dyevaimvga gigvtpyast lvdlvqrtss dsfhrvrcrk vyflwvcsth knyewfvdvl
1381 knvedqarsg llethlfvtq tfhkfdlrtt mlylcekhfr atnsgismft glhaknhfgr
1441 pnfkaffqfl qsehkeqski gvfscgpvnl nesiaegcad anrqrdapsf ahrfetf

SEQ ID NO:31
Human Duox 2 amino acid sequence (residues 1-593, single letter code)

1 mlrarpealm llgalltgsl gpsgsqdals lpwevqrydg wfnnlrhher gavgcrlqrr
61 vpanyadgvy qaleepqlpn prrlsnaatr glaglpslhn rtvlgvffgy hvlsdvvsve
121 tpgcpaefln lrippgdlvf dpdqrgdvvl pfqrsrwdpe tgrspsnprd lanqvtgwld
181 gsaiygsshs wsdalrsfsg gqlasgpdpa fprdsqnpll mwaapdpatg qngprglyaf
241 gaergnrepf lqalgllwfr yhnlwaqrla rqhpdwedee lfqharkrvl atyqnlavye
301 wlpsflqktl peytgyrpfl dpslspefvv aseqffstmv ppgvymrnas chfrkvlnkg
361 fqssqalrvc nnywirenpn lnstqevnel llgmasqlse lednlvvedl rdywpgpgkf
421 srtdyvassl qrgrdmglps ysqallafgl dlprnwsdln pnvdpqvlea taalynqdls
481 qlelllggll eshgdpgplf salvldqfvr lrdgdrywfe ntmglfskk eiedlrntti
541 rdvlvavlni dpsalqpnvf vwhkgapcpq pkqlttdglp qcapltvldf feg

SEQ ID NO:32

Ce. Duox 1 amino acid sequence (residues 1-590, single letter code)

1 mrskhvlyla llfssifggk giqqneefqr ydgwynnlan sewgsagsrl hrdarsyysd
61 gvysvnnslp sarelsdllf kgesglpntr gcttllaffs qvvaheimqs ngvscpletl
121 kiqvplcdnv fdkecegkte lpftrakydk atgnglnspr eqlnertswl dgsflygttq
181 pwvssirsfk qgrlaegvpg yppinnphlp lnnpappqvh rlmspdrlfm lgdsrvnenp
241 gllsfglllf rwhnynanql hrehpdwtde qlfqaarlv iasmqkllay dfvpgllged
301 vrlsnytkym phvppgisha fgaaafrfph slvppamllr krgnkcefrt evggypalrl
361 cqnwwnaqdl vkeysvdell lgmasqlaer ddnlvvedlr dylfgpmhfs rldvvassim
421 rgrdngvppy nelrrtfgla pktwetmned fykkhtakve klkelyggnl lyldayvggm
481 leggengpge lfkellkdqf trlrdgdrfw fenklnglft deevqmihsl tlrdllkatt
541 didetmlqkd vfffkegdpc pqpfqvnttg lepcvpfmqs tywtdndtty

INTERNATIONAL SEARCH REPORT

Inte ›nal Application No
PCI/US 01/15573

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/53 C12N9/02 C07K16/40 G01N33/53

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
BIOSIS, EPO-Internal, SEQUENCE SEARCH, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DUPUY ET AL: "Purification of a novel flavoprotein involved in the thyroid NADPH oxidase" JOURNAL OF BIOLOGICAL CHEMISTRY, AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, BALTIMORE, MD, US, vol. 274, no. 52, 24 December 1999 (1999-12-24), pages 37265-37269, XP002144979 ISSN: 0021-9258 cited in the application the whole document --- -/--	1-3,5-7

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 February 2002	12/03/2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Holtorf, S

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	DUPUY ET AL: " Homo sapiens NADH/NADPH thyroid oxidase p138-tox mRNA, complete cds." EMBL SEQUENCE LIBRARY, XP002144980 accession no. AF181972 ---	1,3,5-7
X	DATABASE EMBL SEQUENCE LIBARY 'Online! 16 May 1997 (1997-05-16) WU X., GRAVES T.: "The sequence of C. elegans cosmid F53G12 - Caenorhabditis elegans cosmid F53G12, complete sequence" XP002190999 accession no. AF003139 ---	5-7
X	DATABASE EMBL SEQUENCE LIBRARY 'Online! 23 January 1998 (1998-01-23) TIN-WOLLAM A., WOHLDMANN P., MORRIS M.: "The sequence of C. elegans cosmid F56C11 - Caenorhabditis elegans cosmid F56C11, complete sequence" XP002191000 accession no. AF043697 ---	5-7
X	DATABASE TREMBL DATABASE 'Online! 1 August 1998 (1998-08-01) TIN-WOLLAM, A., ET AL. : "The sequence of C. elegans cosmid F65C11" XP002191001 accession no. O61213 ---	1-3
P,X	DATABASE EMBL SEQUENCE LIBRARY 'Online! 4 July 2000 (2000-07-04) CHENG G., LEE T., LAMBETH D.: "A bifunctional oxidase (DUOX2) with 77% identity to human DUOX1 and containing regions similar to the superoxide-generating gp91phox and to peroxidases - unpublished" XP002191002 accession no. AF267981 ---	1-7
P,X	DATABASE EMBL SEQUENCE LIBRARY 'Online! 24 May 2000 (2000-05-24) SHARLING L., CHENG G., EDENS W.A., SHAPIRA R., KINKADE J., LEE T., BENIAN G., LAMBETH D.: "Extracellular Matrix Biogenesis by Duox, a Multidomain Oxidase/Peroxidase with Homology to the Phagocyte Oxidase Subunit gp91phox - Caenorhabditis elegans dual oxidase mRNA, complete cds" XP002191003 accession no . AF229855 ---	1-7

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
P,X	DATABASE TREMBL DATABASE 'Online! 1 October 2000 (2000-10-01) SHARLING, L., ET AL. : "Extracellular Matrix Biogenesis by Duox, a Multidomain Oxidase/Peroxidase with Homology to the Phagocyte Oxidase Subunit gp91phox" XP002191004 accession no. Q9NH90 ---	1-3
P,X	WO 00 28031 A (UNIV EMORY) 18 May 2000 (2000-05-18) ---	1-22
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P,X	DATABASE EMBL SEQUENCE LIBRARY 'Online! 7 June 2000 (2000-06-07) DE DEKEN, X., ET AL. : "Homo sapiens NADPH thyroid oxidase2 (thOX2) mRNA, complete cds." XP002191005 accession no. AF230496 ---	1-7
P,X	LAMBETH J D ET AL: "Novel homologs of gp91phox" TIBS TRENDS IN BIOCHEMICAL SCIENCES, ELSEVIER PUBLICATION, CAMBRIDGE, EN, vol. 25, no. 10, 1 October 2000 (2000-10-01), pages 459-461, XP004224276 ISSN: 0968-0004 the whole document ---	1-7
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	EDENS WILLIAM A ET AL: "Tyrosine cross-linking of extracellular matrix is catalyzed by Duox, a multidomain oxidase/peroxidase with homology to the phagocyte oxidase subunit gp91phox." JOURNAL OF CELL BIOLOGY, vol. 154, no. 4, 20 August 2001 (2001-08-20), pages 879-891, XP002190998 ISSN: 0021-9525	

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.1

Although claims 15-18 are directed to a method of treatment - as far as in vivo methods are concerned - of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Although claims 19 - 28 are directed to a diagnostic method - as far as in vivo methods are concerned - practised on the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Continuation of Box I.2

Claims Nos.: 29,30

Claims 29 and 30 relate to a method defined by reference to a desirable characteristic or property, namely by a composition which affects the activity of a duox-protein which is characterized by claims 1-4.

The claims cover all compositions having this characteristic or property, whereas the application provides no support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for such compositions.
In the present case, the claims so lack support, and the application so lacks disclosure, that no meaningful search over the claimed scope is possible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the composition by reference to a result to be achieved.
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The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

formation on patent family members

Int ional Application No
PCT/US 01/15573

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			WO	0149716 A2	12-07-2001

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(26) Publication Language: English

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(63) Related by continuation (CON) or continuation-in-part (CIP) to earlier application:
US 09/437,568 (CIP)
Filed on 10 November 1999 (10.11.1999)

(71) Applicant *(for all designated States except US)*: **EMORY UNIVERSITY** [US/US]; 2009 Ridgewood Drive, Atlanta, GA 30322 (US).

(72) Inventors; and

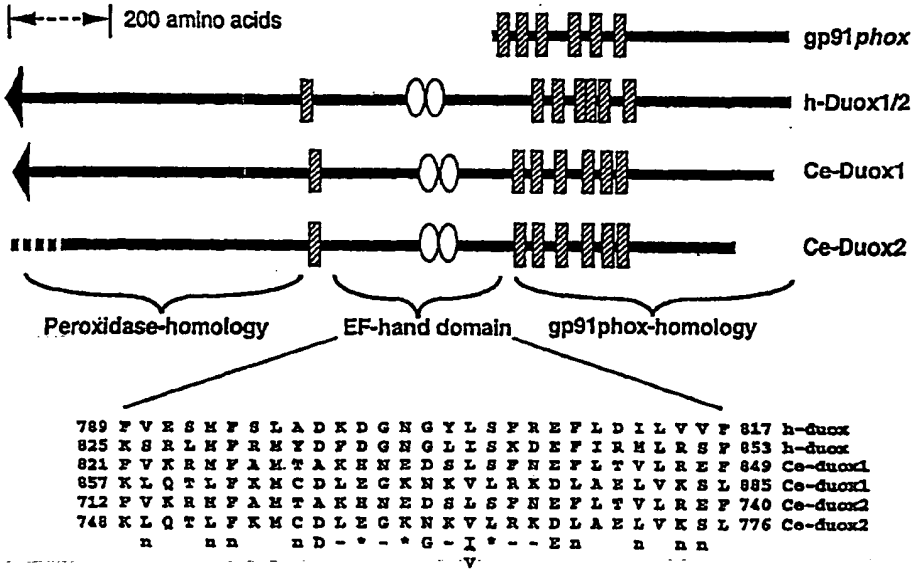
(75) Inventors/Applicants *(for US only)*: **LAMBETH, J., David** [US/US]; 461 Emory Drive, Atlanta, GA 30307 (US). **LASSEGUE, Bernard, P.** [US/US]; 564 Emory Oaks Way, Decatur, GA 30033 (US). **GRIENDLING, Kathy, K.** [US/US]; 1819 Kanawha Trail, Stone Mountain, GA 30087 (US). **ARNOLD, Rebecca, S.** [US/US]; 4344 Bridle Path Court, Tucker, GA 30084 (US). **CHENG, Guangijie** [US/US]; Apartment 1, 2751 Briarcliff Road, Atlanta, GA 30329 (US). **SHARLING, Lisa** [GB/GB]; 2 Limetree Villas, The Chase, Thundersley, Essex 557 3DH (GB). **BENIAN, Guy** [US/US]; 2396 Waterford Cove, Decatur, GA 30033 (US). **EDENS, William, A.** [US/US]; 4171 Hideaway Drive, Tucker, GA 30084 (US).

(74) Agents: **MCDONALD, John, K.** et al.; Kilpatrick Stockton LLP, Suite 2800, 1100 Peachtree Street N.E., Atlanta, GA 30309-4530 (US).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ,

[Continued on next page]

(54) Title: NOVEL DUAL OXIDASES AS MITOGENIC AND ENDOCRINE REGULATORS



(57) Abstract: The present invention relates to new genes encoding for the production of novel proteins involved in generation of reactive oxygen intermediates and in peroxidative reactions that affect biological functions including cell division, thyroid hormone biosynthesis and tissue fibrosis. The present invention also provides vectors containing these genes, cells transfected with these vectors, antibodies raised against these novel proteins, kits for detection, localization and measurement of these genes and proteins, and methods to determine the activity of drugs to affect the activity of the proteins of the present invention.

WO 01/87957 A3

NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

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Published:

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(88) **Date of publication of the international search report:**
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INTERNATIONAL SEARCH REPORT

Inter onal Application No
PCT/US 01/15573

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/53 C12N9/02 C07K16/40 G01N33/53

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
BIOSIS, EPO-Internal, SEQUENCE SEARCH, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DUPUY ET AL: "Purification of a novel flavoprotein involved in the thyroid NADPH oxidase" JOURNAL OF BIOLOGICAL CHEMISTRY, AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, BALTIMORE, MD, US, vol. 274, no. 52, 24 December 1999 (1999-12-24), pages 37265-37269, XP002144979 ISSN: 0021-9258 cited in the application the whole document --- -/--	1-3,5-7

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 February 2002	12/03/2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Holtorf, S

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	DATABASE TREMBL DATABASE 'Online! 1 October 2000 (2000-10-01) SHARLING, L., ET AL. : "Extracellular Matrix Biogenesis by Duox, a Multidomain Oxidase/Peroxidase with Homology to the Phagocyte Oxidase Subunit gp91phox" XP002191004 accession no. Q9NH90 ---	1-3
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	-/--	

INTERNATIONAL SEARCH REPORT

Inter ·ional Application No
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FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

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INTERNATIONAL SEARCH REPORT

Information on patent family members

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